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On the mechanisms underlying neural repression by E(spl)M8 in Drosophila

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ABSTRACT

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Notch signaling is an evolutionary conserved pathway that mediates binary cell-fate specification throughout animal development. Through a process termed lateral inhibition, Notch signaling drives two equipotent cells to adopt distinct fates. Binary cell-fate determination has been exceptionally well studied during Drosophila neurogenesis, particularly, in eye and bristle development. In the eye, Notch mediates the selection of R8 photoreceptors from clusters of R8 precursors by antagonizing the activity of the proneural activator Atonal (Ato). In the bristle, Notch drives the selection of the sensory organ precursors (SOP's) from a group of equipotential cells that expressed the proneural activators encoded by the *achaete scute complex* (*ASC*). In either case, the conserved basic-Helix-Loop-Helix (bHLH) repressors encoded by the *Enhancer of split Complex* (*E(spl)C*) mediate lateral inhibition by antagonizing Ato or ASC. Accumulating evidence indicates that phosphorylation of the E(spl) member M8 by protein kinase CK2 is required for antagonism of Ato/ASC. This modification appears to convert M8 from an autoinhibited state to one that is competent for binding to, and antagonism of Ato or ASC. The work described in this dissertation aims to extend these findings and provide a more detailed understanding of the mechanisms by which M8 mediates neural repression. The studies described in Chapter-2 provide a fundamental reinterpretation of the mechanism by which the *m8* allele *E(spl)D* ablates Ato expression and eye development. Our work indicates that the eye defects of *E(spl)D* reflect the unique biphasic requirements of Notch during R8 specification, where Notch elicits Ato expression and later elicits E(spl) expression. Specifically, we show that the product of *E(spl)D*, a truncated protein called M8*, lacks the autoinhibitory domain thereby allowing it to interfere with the first phase of Notch signaling, itself. As a result, M8* impairs expression of Ato to a level that is insufficient to confer the R8 fate. The work of Chapter-3 provides *in vivo* evidence in support of the autoinhibition model. Using assays for impaired Notch signaling, we show that the C-terminal domain (CtD) of M8 mediates autoinhibition even when expressed as a free peptide. This ability is abolished when the CtD contains a phosphomimetic Asp substitution at the CK2 consensus site, indicating that the 56-residue CtD peptide is sufficient to mediate autoinhibition. Chapter-4 provides genetic evidence that implicates multisite/hierarchical phosphorylation in the regulation of M8 activity. Our studies suggest that CK2 may act as the primary gatekeeper of this cascade of events, which later involve modifications of M8 by MAPK, CK1 and GSK3. Evidence is presented that the MAPK site in M8 is important for neural repression, and that this site is responsive to alteration in EGFR signaling. Multisite phosphorylation may act as a 'timer' controlling the onset of repression, a regulation that is bypassed by the *E(spl)D* mutation. The studies in Chapter-5 demonstrate direct genetic interactions between alleles of *CK2, Notch* and *E(spl)*. The eye, bristle and wing margin defects provide strong evidence that CK2 is a participant in Notch signaling. In Chapter-6, we extend our findings to the bHLH protein Hairy, a member of the HES family. Using in vitro and in vivo assays, we demonstrate that CK2 is required for repression by Hairy as well. Together, the studies described in this dissertation provide novel insights into neural repression, and indicate that posttranslational regulation imposes control over inhibitory Notch signaling.

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Chapter 1

Introduction

The development of metazoan organisms requires a precise spatial and temporal coordination of biochemical and genetic circuits. These circuits involve the participation of diverse signaling pathways that orchestrate the fates of individual cells, thereby enabling organogenesis to occur with precision. Cell-fate determination is one of the most critical steps in metazoan development. The process by which a multicellular organism develops from a single cell, the zygote, is the outcome of toti- or multi-potential cells adopting different and complex identities thereby leading to the formation of organs in the correct position relative to the body plan. In this regard, the biggest challenge is to uncover the mechanisms by which undifferentiated cells respond to dynamic signaling cues that set the stage for the precise development of an organism.

Several decades of research, involving work in diverse animal models, have identified and characterized various aspects of the different signal transduction networks that are responsible for orchestrating development. Collectively, these studies have established that animal development is the result of an intricate interplay of cell proliferation, growth, specification, differentiation, migration, and programmed cell death. Several cell-autonomous and non-autonomous, as well as short- and long-range signals are necessary to guide cells to distinct developmental fates. Surprisingly, these processes are driven by a handful of signaling pathways that are widely conserved through evolution, and operate in different contexts in either a co-operative or antagonistic manner. The challenge before us is to define how these pathways choreograph development, and whether their activities are regulated. Examples of such pathways include, Notch (N), Receptor Tyrosine Kinase (RTK), Wingless (Wnt), Transforming Growth Factor-β (TGF-β), and Hedgehog (Hh). The precise execution of these pathways is necessary for directing differential gene expression, which is critical for proper cell specification during embryonic and later stages of development. Consequently, perturbations in these pathways, either gain or loss of function, lead to severe developmental defects.

Notch and the Epidermal Growth factor Receptor (EGFR) are two such conserved pathways that play critical roles during metazoan development. These two pathways are reiteratively employed in different developmental contexts. This chapter will discuss the architecture of these two pathways, how they mediate signals during development and their intersection. These aspects will focus on Drosophila eye and bristle morphogenesis, as these two developmental paradigms are of most relevance to the studies described in the subsequent chapters.

The Notch signaling pathway:

The Notch signaling pathway is an ancient and evolutionary conserved pathway that is instrumental in the regulation of a number of processes such as proliferation, differentiation, stem cell maintenance, and apoptosis. This pathway mediates short-range communication between adjacent cells, which occurs exclusively in a juxtacrine manner, i.e., through cell-cell interactions via the Notch receptor. As a result of this signaling, two somewhat equipotential cells are forced to adopt different fates. Such binary cell fate determination has been exceptionally well studied in neurogenesis, and this process also operates during myogenesis, and oogenesis (Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000). Although, classically associated with binary cell-fate decisions, Notch is also involved in inductive cell-fate interactions, where patterning occurs through communications between distinct cell types. Examples of Notch mediated inductive signaling can be seen during cone cell patterning in the Drosophila retina, formation of the dorsal/ventral boundary in the Drosophila wing, and during mammalian astrocyte differentiation.

Given its critical roles in diverse developmental contexts, it should not be surprising that anomalies in the Notch pathway have been linked to a number of human diseases and developmental disorders. These include Allagile syndrome (Artavanis-Tsakonas et al.,

1999; Gridley, 2003), familial aortic valve disease (Garg et al., 2005), CADASIL (Artavanis-Tsakonas et al., 1999; Louvi et al., 2006), cancer (Allenspach et al., 2002), multiple sclerosis (John et al., 2002), and congenital heart diseases (High and Epstein, 2008). In addition, Notch has been identified as a therapeutic target for acute T cell lymphoblastic leukemia (T-ALL) (Weng et al., 2004), colon cancer (van ES et al., 2005), and as a potential target for restraining tumor angiogenesis (Noguera-Troise et al., 2006). Because of its involvement in a number of diseases, there is growing interest in uncovering the mechanistic details of the Notch pathway, in basic research, in clinical settings, and for pharmaceutical development.

Thomas H. Morgan first reported the X-linked *Notch* gene in Drosophila in 1916, a nomenclature based on the 'notched' wing phenotype associated with the partial loss of function of this gene. However, its role in development was not recognized for almost 25 years, when David Poulson reported that the complete loss of *Notch* function was associated with embryonic lethality and that these embryos displayed neural hyperplasia (Poulson, 1940). Importantly, and in addition, he demonstrated that neural hyperplasia was closely linked to epidermal hypoplasia, the first indication that this gene may be involved in the assignation of neural versus epidermal cell fates. Later, homologues of Drosophila Notch were also identified in *Caenorhabditis elegans* (Greenwald et al., 1983), and in mammals (del Amo et al., 1993; Lardelli et al., 1994; Uyttendaele et al., 1996; Weinmaster et al., 1992). Unlike the single Notch receptor in Drosophila and C.elegans (Lin-12), mammals contained 4 distinct receptor types, Notch-1, -2, -3, -4. The most detailed mechanistic insights into Notch signaling have emerged from studies in the Drosophila compound eye, a component of the central nervous system, and the mechanosensory bristles, a component of the peripheral nervous system. In a sense, what we have learned about Notch in these two developmental programs may be illustrative of its role elsewhere.

Structure of Drosophila Notch:

Drosophila *Notch* encodes for a 300KDa single pass receptor (Wharton et al., 1985). The extracellular domain contains 36 Epidermal Growth Factor (EGF)-like repeats, which are targets for glycosylation (Fig. 1). These modifications are critical for receptor-ligand interactions, wherein the specificity as well as the affinity of the receptor for its activating ligands is influenced by the type and location of these glycosyl moieties (Lieber et al., 1993). In the case of mammals, some of the EGF-repeats have been reported to bind to Ca^{2+}. This function may influence the structure of the receptor and/or its affinity for ligand binding, thereby influencing signaling (Cordle et al., 2008). This region is followed by three conserved Cys-rich LIN12/Notch Repeats, the LNRs (Lieber et al., 1993). The cytoplasmic region of Notch contains a single RAM domain, which is followed by a long unstructured linker that harbors a nuclear localization sequence (NLS, see Fig. 1). In addition, the linker connects the RAM domain to seven ankyrin repeats, followed by a transcriptional activation domain (TAD) (Fig. 1). The extreme C-terminal region contains a conserved PEST motif, which regulates receptor degradation (Bork, 1993; Breeden and Nasmyth, 1987). Except for some minor differences, the four mammalian receptors are essentially similar to Drosophila Notch. For example, the mammalian Notch 1-4 receptors harbor 36, 36, 34, and 29 EGF-repeats respectively, whereas Notch 3 and 4 lack the TAD domain (Radtke and Raj, 2003).

Components of the Notch pathway:

The basic architecture of the Notch pathway and all of its major components are conserved from Drosophila to mammals. In Drosophila, the pathway consists of the membrane associated receptor Notch, its ligands Delta (Dl) or Serrate (Ser), the downstream component Suppressor of Hairless (Su(H)), and the final effectors the basic Helix-Loop-Helix (bHLH) transcription repressors encoded by the *Enhancer of split Complex*

(*E(spl)C*, Fig. 1). The *E(spl)C* locus in Drosophila is located on the 3rd chromosome and encodes a group of seven bHLH proteins (Mδ, Mγ, Mβ, M3, M5, M7, and M8), and the non-bHLH protein Groucho (Gro) (Delidakis and Artavanis-Tsakonas, 1991; Hartley et al., 1988; Klambt et al., 1989; Knust et al., 1992; Schweisguth and Posakony, 1992). All E(spl) bHLH proteins function as repressors upon interaction with Gro (discussed below).

Glycosylation and receptor maturation:

Notch receptors undergo N- and O-linked glycosylation in the endoplasmic reticulum and Golgi complex, respectively. The EGF-repeats on the extracellular domain of Notch are functionally important targets for glycosylation. Notch is subject to N-linked glycosylation at Asn residues, O-linked glucosylation at Ser residues, and O-linked fucosylation at Ser and Thr residues. These reactions are catalyzed by distinct enzymes, which are located in different compartments. While the importance of O-linked glucosylation remains unclear, O-linked fucosylation is implicated in modulating ligand-receptor interaction, specificity and affinity (Haines and Irvine, 2003; Haltiwanger, 2002; Haltiwanger and Stanley, 2002).

Notch is fucosylated on EGF-repeats harboring the consensus sequence $C_2xxx(A/G/S)(S/T)C_3$ via the activity of O-fucosyltransferase Ofut1 in Drosophila, and Pofut1 in mammals (reviewed in Kopan and Ilagan, 2009). It was initially suggested that this particular modification is necessary to generate a functional receptor, because the loss of Ofut1/Pofut1 elicits phenotypes that mimic strong loss of Notch function in both flies and mice (Okajima and Irvine, 2002). However, later studies in Drosophila have demonstrated that non-fucosylated Notch does move to the cell-surface, bind to ligands, and mediate signals, albeit with reduced potency (Okajima et al., 2008; Stanley, 2007; Vodovar and Schweisguth, 2008).

The importance of glycosylation in Notch signaling has been demonstrated by studies on the glycosyltransferase Fringe (Fng) in Drosophila (Moloney et al., 2000). Studies on Fng reveal that it potentiates differential recognition of ligands by the receptor. Specifically, Fng increases the affinity of Notch for binding Dl, whereas it reduces the Ser-Notch binding (Bruckner et al., 2000; Xu et al., 2007). This differential sensitivity of the Notch receptor towards these two ligands upon modification by Fng is necessary for dorsal/ventral axis formation in flies. In a similar vein, vertebrate homologues of Drosophila Fng modulate Dl-Notch interaction, and inhibit Jagged (Serrate)-Notch interaction (Hicks et al., 2000). A recent study has reported another glycosyltransferase, termed RUMI in Drosophila (Acar et al., 2008). RUMI mediates the addition of O-glucose to specific Ser residues in the extracellular domain of Notch. Unlike other glycosyltransferases, however, RUMI is thought to play direct roles in Notch signaling, as loss of RUMI leads to impaired Notch signaling. However, the effect of RUMI on Notch is temperature dependent and, as such, flies deficient for RUMI's are normal at lower temperature (Acar et al., 2008). The mechanism underlying this temperature sensitive behavior of RUMI remains unclear.

Irrespective of the conservation of the glycosyltransferse enzymes from flies to mammals, the consequences of altered/loss of glycosylations and/or fucosylations are not always identical between these two species. For example, the O-fucosylation site in the EGF repeat-12 is highly conserved, and is critical for mediating interaction of Notch with Dl. This repeat is a target for both Ofut1 and Fng (Panin et al., 2002). In the case of Drosophila, the elimination of EGF repeat-12 leads to a hypersensitive response to Ser, even in the presence of Fng, but does not affect its interaction with Dl (Lei et al., 2003). In contrast, a substitution in an analogous position of the EGF repeat-12 in mouse Notch-1 generates a hypomorphic allele that does not support T-cell differentiation, a Dl dependent process (Ge and Stanley, 2008).

Studies involving different glycosylation mutants have provided further insights into the regulation of Notch. For example mutants harboring missense mutations in EGF repeat-12, N^{M1}, elicits loss of Notch function (de Celis et al., 1993). On the other hand, the *Abruptex* (N^{Ax}) class of alleles harbor point mutations in the EGF repeat-24, 25, 27 and 29, and these display a Notch gain of function phenotypes (de Celis and Bray, 2000; de Celis and Garcia-Bellido, 1994). Another classical mutation, as a result of altered glycosylation of the Notch receptor is the *split* allele (N^{spl}). It harbors an $I^{578}T$ substitution in EGF-repeat 14, which engenders mis-glycosylation, a modification that results in a receptor with greater signaling strength (Hartley et al., 1987; Li et al., 2003). This allele forms the basis of a number of studies (chapters 2, 3, 4, and 5), and will be discussed in greater detail (see below).

Receptor activation:

The activation of the canonical Notch pathway depends upon the interactions between the Notch receptors and its ligands of the DSL (Delta and Serrate in Drosophila, Jagged in mammals and LAG-2 in *C.elegans*) family. Ligand binding leads to the activation of the Notch receptor through cleavage mediated by the ADAM family of metalloproteases (Brou et al., 2000; Mumm et al., 2000). The cleavage site (S2) for the ADAM metalloproteases resides within the LNR region, which prevents Notch proteolysis in the absence of the ligand (Fig. 2). The binding of ligand to the receptor distorts the conformation of the LNR region, thereby exposing the S2 cleavage site. The ADAM protease, Kuzbanian (Kuz), then cleaves the S2 site (Pan and Rubin, 1997). Acute T cell lymphoblastic leukemia (T-ALL) in humans is caused by disruption of this LNR region by point mutations (Weng et al., 2004), resulting in ligand-independent activation of Notch signaling. The S2 cleavage is followed by further proteolysis, the cleavage of Notch

extracellular truncation domain (NEXT) at the S3 site by γ-secretase that finally releases the Notch intracellular domain (NICD) (Okochi et al., 2002; Selkoe and Kopan, 2003). In this context, monoubiquitylation and endocytosis of Notch is essential for γ-secretase activity (Gupta-Rossi et al., 2004). However, it is still unclear whether the plasma membrane or the endocytic compartment is the site for Notch cleavage (Gupta-Rossi et al., 2004; Struhl and Adachi, 2000), or does it occur in both locales, but in different contexts.

Following the release of the NICD by proteolytic cleavage, this fragment translocates to the nucleus via its NLS motif (Hsieh et al., 1996; Lieber et al., 1993). In the nucleus, NICD interacts directly, via its RAM domain (Fig. 2), with CSL (CBF-1 in mammals/Su(H) in Drosophila/LAG-1 in *C.elegans*) family of transcription factors (Bailey and Posakony, 1995; Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995). CSL proteins then drive transcription of the terminal effectors, the bHLH E(spl)/HES repressors. In the absence of the NICD, Su(H) acts as a transcriptional repressor of these Notch target genes, through the co-repressor protein Hairless (H) (Schweisguth and Posakony, 1994). This view is supported by studies showing that loss of *H* elicits ectopic and inappropriate activation of the Notch targets genes (Barolo et al., 2000; Furriols and Bray, 2000). H functions along with the co-repressors C-terminal binding protein (CtBP) and Groucho (Gro) by binding to DNA-bound Su(H) (Barolo et al., 2002). Histone deacetylases (HDACs) are recruited to the Su(H)/H/CtBP/Gro complex to actively maintain *E(spl)/HES* in a repressed state (Lai, 2002). The conversions of Su(H) from a repressor to activator is facilitated by NICD along with other co-activators such as Mastermind (Mam) in Drosophila or Mam-like (MAML) in mammals (Petcherski and Kimble, 2000). Mam, in turns, recruits p300, and other histone acetyl transferases (HAT's). This favors the assembly of the transcriptional complex that activates expression of the Notch target genes (Wallberg et al., 2002). Analysis of the crystal structure of the Notch/Su(H)/Mam ternary complex suggests that conversion of Su(H)

from a repressor to an activator occurs due to a conformational change induced by cooperative interaction of Su(H) with NICD and Mam (Barrick and Kopan, 2006; Wilson and Kovall, 2006).

Notch signaling during Drosophila neurogenesis:

In Drosophila, the precise interplay between two families of bHLH transcription factors is central to the process of neurogenesis. These two groups are the proneural activators, encoded by *atonal (ato)* and the *achaete-scute complex (ASC),* and the repressors encoded by *E(spl)C*. While *ato* is required for eye development, the *ASC* members drive bristle morphogenesis (Campos-Ortega, 1997; Dambly-Chaudiere and Vervoort, 1998; Massari and Murre, 2000). Irrespective of the developmental settings, the basic mechanism underlying the interplay between these activators and repressors is somewhat similar.

The onset of neural cell-fate specification involves the expression of *ASC/ato* that bestows on cells the ability to become neural precursors. The activities of these transcription factors are essential for the formation of a group of equipotent cells termed as the proneural cluster (PNC's). However, from each PNC, only a single cell goes on to forms the neuronal cell, which has been termed the sensory organ precursor or SOP in the case of the mechanosensory bristles, and the R8 photoreceptors in the case of the eye. The process of selection of the SOP/R8 initiates when one cell of the PNC gains an advantage over the others by virtue of producing the highest level of ASC/Ato. The high levels of proneural activators drive cell-cell communications between the future SOP's/R8's and its immediate neighbors in a PNC. This communication involves an inhibitory signal that is mediated by Notch (Artavanis-Tsakonas et al., 1999; Baonza and Freeman, 2001; Bray, 1997; Mumm and Kopan, 2000). Specifically, the cell destined to become the SOP/R8

expresses the ligand Dl at a level sufficient to activate Notch in the adjacent cells within the PNC. Activation of Notch in the non-SOP's, the signal receiving cells, leads to the cleavage and translocation of NICD (Schroeter et al., 1998) to the nucleus, where it switches Su(H) into a transcriptional activator (Fig. 3). This leads to transcription of the E(spl) repressors (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). The E(spl) repressors then complex with the ubiquitously expressed co-repressor Gro to antagonize the activities of ASC/Ato. In contrast, the cell destined to become the SOP/R8 remains refractory to inhibitory Notch signaling, as Su(H) in combination with Hairless (H) and Gro maintains the *E(spl)* locus in a quiescent state (Castro et al., 2005; Hinz et al., 1994; Koelzer and Klein, 2003). As a result, Notch signaling rapidly leads to the extinction of Ato/ASC in all but the future R8/SOP, leading to the patterning of these neural founder cells.

Notch signaling during eye development:

The Drosophila eye is an excellent developmental context to define the mechanisms by which signaling pathways orchestrate organogenesis. The compound eye of Drosophila is composed of ~800 light sensing facets called ommatidia. Each ommatidium is composed of eight photoreceptors, the retinula cells R1-R8, and 12 accessory non-neuronal cells such as the pigment cells that optically shield the ommatidia from each other and cone cells that form the lens (Freeman, 1997). The ommatidia are arranged in a precise hexagonal lattice, with interommatidial bristles (IOB's) at alternating vertices of each ommatidium (Fig. 4). Any perturbations in the specification of the aforementioned cell fates disrupt the hexagonal patterning of the adult eye or the positioning of the IOB's, and are easily observable by light microscopy.

The specification of the R8 cells is a critical and essential first step during eye development, as this cell type orchestrates the specification of all retinal cell fates, neuronal

and non-neuronal, and is therefore vital for establishing the precise architecture of the adult eye. These include the secondary photoreceptors (R1-7), the cone cells and the pigment cells (Frankfort and Mardon, 2002; Hsiung and Moses, 2002; Jarman et al., 1994; Kumar and Moses, 2001). Thus precise R8 patterning is vital for eye development. Because of this critical role played by the R8 cells in ommatidial formation, they are referred to as the 'founding' photoreceptors. Owing to the centrality of the R8's in mediating retinal development, extensive studies have been directed at uncovering the underlying mechanisms.

The mechanism underlying R8 specification:

Eye morphogenesis initiates in the eye imaginal disc, a monolayer of multipotential neuroepithelial cells, which arise from ~6 cells set aside in the early embryo. During the first and second larval stages these cells divide to give rise to the eye anlagen. R8 differentiation begins in the third larval stage with the appearance of an apico-basal indentation at the posterior margin of the eye disc. This indentation, termed the morphogenetic furrow (MF), moves across the eye disc towards its anterior margin over a period of 48 hours (Ready et al., 1976). The MF marks the boundary between the cells that are unspecified and undifferentiated (anterior to MF), from those that are undergoing specification and differentiation (posterior to MF). The MF is thus the region where cell-fate specification initiates in a temporally and spatially ordered manner along the DV axis, such that photoreceptor clusters in one column are out of phase with those in the adjacent columns (Fig. 4). The initiation of the MF and the specification of retinal cell-fates involve signaling pathways such as Wnt/wingless, Notch, EGFR, etc. While the mechanism by which the MF is formed remains an enigma, the progression of this wave of differentiation appears to require Hh, which is secreted by differentiating photoreceptors (Heberlein and

Moses, 1995; Ma et al., 1993). This raises the quandary that no differentiated photoreceptors are present in the eye disc prior to the formation of the MF, itself. What is well known, however, is that Hh induces long-range signaling by the secreted protein Decapentapelagic (Dpp), which upregulates the expression of *ato*, *hairy* (*h*) and *extramacrochaete* (*emc*). The latter two proteins serve to restrict the expression domain of *ato* to the anterior margin of the MF (Brown et al., 1995; Fu and Baker, 2003; Greenwood and Struhl, 1999), where specification of the first photoreceptor, the R8 cell, occurs.

R8 patterning occurs in the MF and involves the biphasic Notch functions that are separated in time and space (Ligoxygakis et al., 1998). The process of R8 specification is divided into four distinct stages (Fig. 4). In cells at the anterior margin of the MF (stage 1), Notch elicits *ato* expression, initiated by Hh and Dpp (Greenwood and Struhl, 1999; Sun et al., 1998) in a generally uniform manner along the DV axis of the eye disc (Baker et al., 1996; Baker and Yu, 1997). During this stage, Notch also negatively regulates the expression of *h* and *emc* (Baonza and Freeman, 2001). This results in the upregulation and accumulation of Ato in all cells at stage 1. This expression is necessary to maintain neural competency of these cells, the first step in the formation of the PNC's. This stage of Notch signaling has been termed ‘proneural enhancement’, and is Su(H)- and E(spl)-independent (Baker et al., 1996). As Ato levels rise, a positive feedback loop (proneural self-stimulation) leads to uniform and high Ato levels. The distinction between proneural enhancement and proneural self-stimulation reflects 3' and 5' enhancers of *ato* (Sun et al., 1998).

Lateral inhibition then resolves this uniform expression of *ato* to specify single R8 cells, which emerge in a phase shifted manner at the posterior margin of the MF. This process occurs at stage 2/3. During lateral inhibition, one cell from each PNC gains an advantage over the others by expressing the highest amount of Ato. As a result, this pro-R8 cell initiates Notch-dependent lateral inhibition (Sun et al., 1998). Specifically, the pro-R8

cell expresses the Notch ligand Dl at a level sufficient to activate Notch in the neighboring cells. Activation of Notch leads to the cleavage and translocation of NICD to the nucleus, where it mediates the expression of the E(spl) repressors, which then antagonize the activity of Ato (Bailey and Posakony, 1995; Ligoxygakis et al., 1998). This phase of Notch signaling is Su(H)- and E(spl)-dependent. Of the seven *E(spl)* genes, only *m8*, *mγ* and *mδ* are expressed in the MF (Cooper et al., 2000). However, *m8* is thought to play a predominant role in R8 selection, because its overexpression and mutation in the *E(spl)D* allele severely attenuates retinal patterning. In contrast, the loss of either Mγ or Mδ does not affect eye development with equal severity (The et al., 1997).

A body of evidence supports this biphasic nature of Notch during R8 specification. For example, an absence of Notch during 'proneural enhancement' abrogates *ato* expression, and leads to a loss of all R8 cells (Cadigan and Nusse, 1996; Cagan and Ready, 1989; Frankfort and Mardon, 2002; Jarman et al., 1994), thereby leading to a complete loss of the eye field akin to that in *ato*^{1} (a null allele) flies. In contrast, an absence of Notch during lateral inhibition leads to the specification of supernumerary R8 cells (Ligoxygakis et al., 1998). These excess R8's compromise retinal patterning and manifest in the adult as a rough eye. This model of Notch function is further supported by the observation that loss of Su(H) attenuates E(spl) expression and elicits supernumerary R8's, but does not affect *ato* expression at stage 1 (Koelzer and Klein, 2003).

Lateral inhibition therefore allows for precise positioning of R8's in the developing retina, and at the same time ensures that cells that received the Notch signaling remain uncommitted to the neural fate. This, in turn, allows for the recruitment of these cells as secondary photoreceptors, a process that only initiates at stage 4 (Jarman et al., 1994). The recruitment of these secondary photoreceptors is highly ordered and invariant. The differentiated R8 recruits R2/5, R3/4, R1/6, R7, cone cells and the pigment cells (Fig. 4) in

an ordered manner (Cagan and Ready, 1989). Notch also plays critical roles in the specification of these later cell fates (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Flores et al., 2000; Nagaraj and Banerjee, 2007; Tomlinson and Struhl, 2001).

Notch signaling during bristle development:

Macrochaetes (MCs) constitute a major group of mechano-sensory organs of the peripheral nervous system. The number and positions of these bristles are invariant and are also regulated by the Notch pathway (Hartenstein and Posakony, 1990; Heitzler et al., 1996; Modolell and Campuzano, 1998). Each bristle consists of four distinct cell types; a neuron, sheath, shaft, and socket that arise from a single precursor cell termed the sensory organ precursor (SOP's, Fig. 5). Although specification of the SOP's occurs in the third instar larva, bristle morphogenesis does not commence until the pupal stage. Each bristle develops from an SOP that is selected from a group of equipotent cells, the PNC's (Heitzler et al., 1996; Jarman et al., 1995). The most detailed analysis has involved the specification of bristles on the thorax. The PNC's that give rise to these bristles are formed at precise positions in the wing imaginal disc, the tissue from which thorax arises. Initially, all cells of the PNC express the *ASC* bHLH transcription activators (Cubas et al., 1991). Unlike *ato,* however, expression of *ASC* is not dependent on Notch, but instead depends on pre-pattern factors (Calleja et al., 2002).

In a process that is thought to be stochastic, one cell from each PNC gains an advantage over the others by producing the highest levels of ASC. This cell is destined to become the SOP, and initiates lateral inhibition in the neighboring cells (Artavanis-Tsakonas et al., 1999; Castro et al., 2005; Giebel and Campos-Ortega, 1997; Lai, 2004). The SOP then goes onto form the pI neuroblast. The pI neuroblast divides asymmetrically to give rise to the pIIa and pIIb cells (Fig. 5). Asymmetric division of the pIIa cell gives rise to the socket

and shaft cells, which form the external structures of the bristle. In contrast, the pIIb cell divides asymmetrically to give rise to the glial cell and the third order precursor, the pIIIb cell. Asymmetric division of pIIIb gives rise to the sheath and the neuron, which are internal components of the bristles. Each of these divisions, which is coordinated by Notch and E(spl), ensures the stoichiometric specification of the four sister cell fates (socket, shaft, neuron and sheath) that comprise the bristle.

A body of genetic evidence supports the reiterated deployment of Notch signaling in bristle development. For example, a loss of Notch functions prior to SOP selection results in the specification of extra SOPs. Each of these goes on to form a bristle, and consequently manifests as ectopic closely spaced bristles in the adult (Hartenstein and Posakony, 1990). On the other hand, if Notch is lost after SOP selection has been completed, the stoichimetric specification of the four sister cell fates is skewed (Bray, 1997; Campos-Ortega, 1997), and manifest as split or missing bristles (Bray, 1997; Campos-Ortega, 1997). A split bristle reflects a defect in division of the pIIa cell, which leads to the transformation of the socket cell into a shaft cell. On the other hand, a missing bristle can result either from the loss of SOP or from a defect in the asymmetric division of the SOP. While the former can reflect excessive E(spl) activity, the later is the outcome of a defect in the asymmetric division that normally generates one pIIa and one pIIb cell. Instead, two pIIb cells arise, and go on to specify twinned neurons and sheath cells. Because the external shaft and socket cells are missing, this manifests as a missing bristle.

The *split* allele of *Notch*:

One of the classic mutations of Notch is the *split* allele (N^{spl}). N^{spl} is a viable and recessive mutation of *Notch* that harbors an $I^{578}T$ substitution in its 14^{th} extracellular EGF-repeat (Fig. 6). The substitution introduces an extra glycosylation site, whose modification

alters Notch sensitivity to its ligands (Hartley et al., 1988; Li et al., 2003). Females heterozygous for N^{spl} do not exhibit any overt phenotypes, except for subtle bristle abnormalities such as, missing or duplicated MCs. In contrast, hemizygous (N^{spl}/Y) males are characterized by a rough and reduced eye, and extensive bristle defects such as, split, missing, and duplicated MCs.

There are two schools of thoughts on the mechanism(s) underlying the neural defects of N^{spl}. Preiss and co-workers argue that N^{spl} is a loss of function mutation (Nagel and Preiss, 1999). Specifically, they suggest that the retinal defects of N^{spl} reflect attenuated Notch activity during the 'proneural enhancement' stage that is required to establish the initial expression of Ato. Loss of Notch at this stage is expected to compromise Ato levels, negatively impact R8 specification, thereby leading to the rough and reduced eye. In contrast, Baker and co-workers have suggested that N^{spl} is a gain of function mutation (Fig. 6). They find that N^{spl} renders R8 precursors sensitive to Dl, and thus to inhibitory Notch activity. In this case, this inappropriate Notch activity leads to aberrant expression levels of Ato, which compromises expression of the Ato target gene *senseless* (*sens*). Sens is necessary for differentiation of the R8 precursors into R8 photoreceptors. Because of this defect, many R8 cells undergo apoptosis (the default), and the few R8's that survive are themselves compromised for secondary photoreceptor specification. This results in defects in secondary photoreceptor recruitment and manifests as ommatidia of varying sizes. Moreover, Hh secretion by the differentiated photoreceptors is deficient, thereby compromising further R8 recruitment. The loss of Ato in the presumptive R8's is therefore not a consequence of compromised proneural enhancement but rather reflects non-autonomous effects.

An examination of the literature would support the proposal of Baker and coworkers. N^{spl} flies have been screened for both enhancers and suppressors. Most relevant are the

observations that the reduced and rough eye of N^{spl} are rescued by reduced dosage of *E(spl)* or *Dl* (Shepard et al., 1989). Under these conditions, inhibitory Notch signaling would be diminished in the sensitized R8 cells thereby allowing for accumulation of Ato to a level sufficient to confer the R8 fate. In addition, mutations in *ato* or its heteromeric coactivator *da* further exacerbate the rough and reduced eye of N^{spl}. This finding is consistent with the proposal that the Ato defect in N^{spl} is non-autonomous (Parks et al., 1995; Kahali et al., 2009).

In addition to the eye, N^{spl} also perturbs bristle patterning, indicating that this mutation also enhances inhibitory Notch signaling in the SOP lineage. Surprisingly, even though Notch is involved in other developmental contexts, N^{spl} does not affect wing morphogenesis, oogenesis, and myogenesis, and consequently these flies are normal for viability and fertility.

The bHLH E(spl) repressors:

As mentioned above, E(spl) proteins are the final effectors of inhibitory functions of Notch signaling during both eye and bristle development. In either case, these proteins antagonize Ato/ASC during the execution of lateral inhibition and, therefore, restrict neural competency (provided by Ato/ASC) to a single cell from each PNC's. The *E(spl)C* is located on the 3rd chromosome in Drosophila, and encodes seven bHLH proteins (Mδ, Mγ, Mβ, M3, M5, M7, and M8), and the non-bHLH protein Gro (Delidakis and Artavanis-Tsakonas, 1991; Knust et al., 1992). Surprisingly, the density and syntenic order of these eight transcription units has remained invariant during Drosophila evolution (Maier et al., 1993). This extraordinary conservation raises the possibility that these proteins are unlikely to be functionally redundant, a prediction that appears to bear out with more detailed analysis of individual E(spl) members (see below).

Over the years, extensive studies have been directed at uncovering the mechanisms underlying neural repression by E(spl) proteins. These studies have identified multiple functional domains (Fig. 7). These include an N-terminal basic domain (DNA binding), a helix-loop-helix (HLH) domain (homo/hetero-dimerization amongst E(spl) members), a second HLH domain termed Orange (specificity for interaction(s) with Ato/ASC), and a C-terminal WRPW motif (Gro-binding). The one region where E(spl) members diverge from each other is that between Orange and WRPW. This region, which we called the C-terminal domain (CtD), predominantly accounts for the length and sequence differences between E(spl) proteins. Consequently, the CtD has been thought to not be vital for repression, but studies conducted by our laboratory indicate that it is, in fact, this region that may underlie non-redundancy amongst the E(spl) members. In the case of M5, M7, and M8, the CtD harbors a consensus site for phosphorylation by CK2 (Fig. 7) raising the possibility that repression is a controlled process (Trott et al., 2001).

Mechanisms of neural repression by E(spl) proteins:

It was initially proposed that E(spl) proteins mediate repression by binding to the regulatory regions of proneural genes (Van Doren et al., 1994). This suggestion reflected the highly conserved basic region in all E(spl) proteins, and the presence of a binding element, called the N-box, in the enhancer of *ASC* (Oellers et al., 1994). Additionally, E(spl) proteins bind DNA in vitro, an interaction that requires a functional basic domain. In addition, reporter constructs that harbor an N-box sequence are potently repressed by ectopically expressed E(spl) proteins (Tietze et al., 1992; Van Doren et al., 1994). Moreover, studies with a chimera between M7 and the transcriptional activation domain of VP16, called $M7^{Act}$ (Jimenez and Ish-Horowicz, 1997), converted M7 from a repressor to a potent transcriptional activator. Furthermore, this activity of $M7^{Act}$ was attenuated by

mutation of key residues, or deletion of, the basic domain. These observations led to the view that E(spl) proteins function as a enhancer-bound repressors in vivo.

More recent studies have questioned a role for DNA binding. Foremost among these was the observation that deletion of the basic domain or its neutralization did not compromise the ability of ectopically expressed variants of M7 or M5 to block bristle development (Giebel and Campos-Ortega, 1997; Nakao and Campos-Ortega, 1996). These observations began to question if DNA binding was essential for repression. It has subsequently been shown that deletion of the N-box from the *ASC* enhancers does not impair repression in vivo. Moreover, the enhancers of *ato* lack any bonafide N-box like sequence, suggesting that alternative mechanism underlie repression.

A more recent model, one supported by both in vivo and in vitro evidence, is that repression reflects direct interaction of E(spl) proteins with enhancer bound Ato/ASC activators. This model, termed the protein tether, would account for the high specificity and affinity interactions of E(spl) proteins with Ato/ASC at the protein level (Gigliani et al., 1996; Nagel et al., 1999). These interactions are thought to involve the Orange domain of E(spl) proteins (Dawson et al., 1995; Giebel and Campos-Ortega, 1997).

The classical view of the role of E(spl) in Notch signaling:

Studies involving bristle and SOP development (Giebel and Campos-Ortega, 1997; Nakao and Campos-Ortega, 1996; Skeath and Carroll, 1991; Tata and Hartley, 1995) have led to the notion that transcription and accumulation of E(spl) proteins was, by itself, sufficient for neural repression. However, studies in the eye indicate otherwise. In this case, loss of E(spl) did compromise lateral inhibition and result in excess R8's, but ectopic expression of M8, M5, M7 did not elicit a dominant loss of the R8 cells (Ligoxygakis et al.,

1998). It could be argued that the inactivity of M5 and M7 may reflect the fact that these two members are not expressed during R8 patterning (Cooper et al., 2000). In contrast, endogenous M8 is expressed in the MF and its mutation, the *E(spl)D* allele, severely affects R8 patterning (see below). Consequently, it was suggested that M5 and M7 are qualitatively different than M8, but the nature of this difference remained unresolved.

The unique dominant allele of *m8, E(spl)D*:

The importance of E(spl) for lateral inhibition is best illustrated by the dominant allele *E(spl)D*, which was identified based on its ability to abrogate retinal development in the presence of N^{spl}. It was, in fact, this genetic interaction that led to the identification of the *E(spl)C* (Welshons, 1956). In the 50 years hence, *E(spl)D* still represents the only mutation in an individual E(spl) member. *E(spl)D* is characterized by two molecular lesions. The first of these is a nonsense mutation that terminates translation at Leu^{123}, immediately after the Orange domain. Consequently, the truncated protein, called M8*, lacks the 56 residues C-terminal domain (CtD). On the other hand, the second lesion affects an element in the 3' UTR of the *m8* transcript that is required for transcript turnover (Klambt et al., 1989; Preiss et al., 1988), thereby leading to accumulation of the mutated transcript (Tietze et al., 1992).

Follow-up studies sought to determine which of these lesions contribute to the dominant eye defects of *E(spl)D*. It was found that overexpression of a *UAS-m8** construct recapitulated the in vivo effects of *E(spl)D*, but no such effects were seen upon overexpression of full-length M8. These findings indicated that the absence of the CtD was somehow mediating the enhancement of N^{spl} (Tietze et al., 1992). Because the CtD removes the WRPW motif, it has been suggested that *E(spl)D* is a 'Gro-independent hypermorph' (Nagel et al., 1999). While this proposal would be consistent with the findings, it remained a paradox because removal of the WRPW motif from otherwise full-length M8

renders the protein non-functional, an outcome also seen with the related repressor Hairy. To address this paradox, the late Dr. Campos-Ortega presciently proposed that the CtD of M8 might play a regulatory role, but the biochemical mechanism remained unclear, until it was uncovered that this region was a target for phosphorylation (Trott et al., 2001).

Repression by M8 is phosphorylation dependent:

In 2001, it was found that protein kinase CK2 interacted with M8, M5, and M7 (Trott et al., 2001). These three proteins harbored a conserved CK2 recognition motif in the CtD (Fig. 7). In the case of M8, Ser^{159} was identified to be the sole target for phosphorylation by CK2, because its replacement with either Ala or Asp abolished phosphorylation.

The role of CK2 mediated phosphorylation in repression by M8 has been studied by the binary Gal4-*UAS* system (Karandikar et al., 2004). It was found that expression of a CK2 phosphomimetic *UAS-m8SD* construct (replaces Ser^{159} with Asp) led to a severely reduced eye, a phenotype that closely mimicked the retinal defects of *E(spl)D* M8*. No such effects were seen with wild-type M8 or the non-phosphorylatable variant M8SA (replaces Ser^{159} with Ala) indicating that the CK2 site does regulate repression by M8 (Karandikar et al., 2004). In the case of M8SD, it was shown that the reduced eye reflected loss of Ato-positive cells, i.e. the founding R8's (Karandikar et al., 2004). This loss of R8's precipitates apoptosis in all cells posterior to the MF, an outcome similar to that upon loss of Ato (Jarman et al., 1995; White and Jarman, 2000). These effects of M8SD are virtually identical to those reported for *E(spl)D*. Furthermore, M8SD interacted with Ato with an affinity comparable to that of M8*. No such interactions with Ato could be detected with wild type M8 or M8SA. These studies indicated that the retinal defects of M8SD and M8* are mechanistically similar and are Ato-specific. How might one reconcile the similar outcomes of a truncated protein lacking the phosphorylation site (M8*) and a phosphomimetic full-

length variant (M8SD)? It was proposed that M8 is autoinhibited by its CtD in an intramolecular manner. This *cis*-interaction could occur through the first HLH or the Orange domain. Consequently, non-phosphorylated M8 would be prevented from binding to Ato, an interaction that appears to be essential for repression. In this case, phosphorylation would displace the CtD to expose Orange and enable binding to Ato and repression of R8 fate. It was thus proposed that the truncation of the CtD in M8* removed this regulatory region, thereby resulting in a protein that constitutively binds to Ato. If so, repression by M8 is regulated by posttranslational modification and suggest that CK2 is a participant in inhibitory Notch signaling (Karandikar et al., 2004). The lack of suitable antibodies has precluded a direct assessment of M8 phosphorylation during R8 refinement. Unlike the eye, however, M8 inhibited bristle development, leading to the suggestion that the role of CK2 might be dispensable in antagonism of ASC (Karandikar et al., 2004).

Follow-up studies to assess the role of CK2 have employed targeted misexpression of CK2-RNAi or CK2-DN constructs during R8 and SOP selection (Bose et al., 2006). It was found that compromising CK2 levels/activity lead to a rough eye phenotype that reflected the specification of supernumerary R8's, supporting studies with the CK2 mimetic M8SD protein (see above). Moreover, this defect in R8-specification has been described upon loss of E(spl) (Ligoxygakis et al., 1998). Unexpectedly, it was also found that compromising CK2 levels/activity led to ectopic, split, and missing MC's on the thorax, raising the possibility that CK2 also plays a role in the SOP lineage. Importantly, these bristle defects were enhanced by a reduction in the dosage of the *E(spl)C*, indicating that these bristle defects may reflect the hypophosphorylation of endogenous E(spl) proteins.

Protein Kinase CK2:

CK2 is a conserved and ubiquitously expressed Ser/Thr protein kinase. This enzyme plays critical roles in diverse cellular and biochemical events that include cell cycle progression, DNA replication, DNA repair, transcription, translation, apoptosis (Glover, 1998; Pinna, 1994).

The laboratory of Eugene Kennedy was the first to describe the presence of an enzyme, which they called a protein 'phosphokinase'. This enzyme catalyzed the transfer of the γPO4 group of ATP to the model substrate casein (Burnett and Kennedy, 1954). In addition, they demonstrated that this enzyme modified Ser/Thr residues, the first demonstration for the existence of a protein kinase. Unable to associate a biological or biochemical effect on Casein, Eugene Kennedy did not further pursue work on this 'enzyme'.

It was not until the seminal work of Edmond Fischer and Edwin Krebs that the significance of protein phosphorylation became apparent. In their landmark studies, Fischer and Krebs demonstrated that phosphorylation converted the enzyme glycogen phosphorylase from an inactive to an active form (Fischer and Krebs, 1955). Specifically, they demonstrated that activation of phosphorylase was catalyzed by cAMP dependent protein kinase (PKA). This was the first demonstration that the activities of proteins are fundamentally altered by the simple addition of one phosphate group. The importance of their finding is underscored by whole genome sequencing effort, which indicates that eukaryotic genomes encode in excess of 400-500 distinct protein kinases. These kinases control all aspects of eukaryotic cell biology such as cell division, DNA replication, transcription, translation, cytoskeletal structure, cell polarity, secretion, nuclear import, protein degradation, etc. Studies in metazoan models have demostrated that in addition to cell autonomous functions, protein kinases are vital for all aspects of development; from early embryogenesis through post-natal development.

The laboratory of Jolinda Traugh was the first to isolate and purify mammalian Casein Kinase. During purification, they identified two forms of the enzyme, and named them Casein Kinase I (CKI) and Casein Kinase II (CKII) based on their order of the elution in column chromatography (Hathaway and Traugh, 1979). Following purification, antibodies against the mammalian enzyme were generated and found to recognize, with high affinity and specificity, the homologous enzyme in tissue extracts from organisms such as Drosophila and worms (Dahmus et al., 1984). These findings indicated that this enzyme is highly conserved. Subsequently, CKII was purified from Drosophila, worms, and yeast. Over the years, extensive biochemical, molecular, and genetic analysis have revealed that CKI and CKII are distinct groups of enzyme with distinct biological functions. Given their importance in cell and organismal biology, and that casein is not a physiological substrate, these two enzymes were renamed as protein kinase CK1 and CK2.

Subunits of CK2:

The CK2 holoenzyme is a heterotetramer (Fig. 8) that is composed of two catalytic (α) subunits and two regulatory (β) subunits (Glover, 1998; Glover et al., 1983; Pinna, 1990; Pinna, 1994). Both, the subunits as well as the tetrameric conformation of CK2 are conserved throughout evolution. The mammalian enzyme contains two distinct isoforms of the catalytic subunit (α and α') that are encoded by distinct genes, and a single gene that encodes for the regulatory CK2β subunit. In contrast, Drosophila contains a single CK2α isoform, but exhibits the presence of three distinct genes (β, β', and *SSL*) encoding for regulatory CK2β subunits (Bidwai et al., 1999; Karandikar et al., 2003).

The tetrameric conformation of CK2 resembles the ternary state of PKA, which also exists as a tetramer of two regulatory (R), and two catalytic (C) subunits (Taylor, 1989). In

the case of PKA, cAMP binds to the R subunits and triggers the dissociation of the C subunits, which are then switched into an active conformation. In contrast, the tetrameric conformation of CK2 is highly stable. Using resolution and reconstitution, Cochet and Chambaz found that monomeric CK2α displayed catalytic activity, albeit at levels that were 20% of those seen with the tetramer (Cochet and Chambaz, 1983). Furthermore, the addition of CK2β reconstituted the tetrameric state and full catalytic activity. This observation has since been confirmed using recombinant proteins expressed in insect cells, bacteria, or yeast (Bidwai et al., 1992b; Birnbaum et al., 1992; Boldyreff et al., 1993). Collectively, these results suggest that even if a second messenger were to trigger dissociation of the CK2 holoenzyme in vivo, it could attenuate CK2 activity only five fold. Such a modest level of regulation would appear to be incompatible with the notion that protein kinase activities are tightly regulated in vivo.

Biochemistry of CK2:

CK2 can utilize either ATP or GTP as a phosphoryl donor (Dahmus et al., 1984; Glover et al., 1983; Hathaway et al., 1980; Meggio et al., 1982). The laboratory of Edwin Krebs identified that CK2 recognizes a unique consensus sequence; S/T-D/E-x-D/E, where x represents any amino acid, except K/R (Kuenzel et al., 1987). The acidic residues at positions n+1 and n+3 are rate limiting for phosphorylation (Kuenzel and Krebs, 1985; Kuenzel et al., 1987). This consensus reveals that CK2 preferentially phosphorylates S/T in acidic microdomains, a characteristic that is unique among the Ser/Thr kinase family. Additional acidic residues at the N- and C-terminus of this consensus further enhance phosphorylation. In addition, the effect of these acidic residues can be biochemically mimicked by pSer/pThr, raising the possibility that CK2 can operate in hierarchical phosphorylation cascades.

Drosophila CK2:

The catalytic subunit is encoded by the *CK2α* gene located on the 3rd chromosome (Saxena et al., 1987), whereas *CK2β* is located on the X chromosome. A mutation in *CK2α*, *Timekeeper (Tik)*, was identified based on its ability to elicit dominant defects in the circadian clock (Lin et al., 2002). *Tik* harbors two missense mutations, $M^{161}K$ and $E^{165}D$ (Fig. 8). The $M^{161}K$ substitution resides in the ATP-binding pocket, thereby abrogating enzyme activity. Accordingly, *Tik* is homozygous lethal and lethality manifests at the first larval instar. In contrast, the second substitution, $E^{165}D$ was thought to be silent because it involved a conservative replacement. Sequence analysis, however, reveals that this substitution resides in a highly conserved motif, $HE^{165}NRKL$, which mediates interaction between human CK2α and protein phosphatase, PP2A in human (Heriche et al., 1997). Later, studies in Drosophila have also implicated a similar interaction. Therefore, it has been proposed that *Tik* is a 'double hit', on one hand, it inactivates the kinase activity, and on another, it leads to enhanced PP2A activity (Kunttas-Tatli et al., 2009).

A spontaneous revertant of *Tik*, called *TikR* was identified based on partial reversal of the clock defects of *Tik/+* animals. In addition to the two lesions associated with *Tik* (see above), *TikR* harbors a deletion of seven amino acids (234-240) and the substitution of Arg^{242} with Asp (Fig. 8). Consequently, *TikR* is also catalytically dead, and is lethal when homozygous. However, apart from clock defects, neither *Tik* nor *TikR*, elicit any overt neural (eye/bristle) defects in the heterozygous condition (Bose et al., 2006).

Biochemical studies indicate that TikR is a misfolded protein, a defect not seen with Tik (Lin et al., 2002). In addition, it has also been shown that unlike Tik or CK2α, TikR does not interact with CK2β (Kunttas-Tatli et al., 2009). Based on these observations, it appears that Tik integrates into the tetrameric holoenzyme, and consequently, 'poisons' the

holoenzyme. In contrast, the inability of TikR to assemble into the holoenzyme may be the reason for its revertant behavior in the context of the circadian clock.

Recently, another allele of *CK2*α, *CK2*^{MB00477} (Fig. 8) has been described (Bellen et al., 2004). It harbors a transposable *minos* element in the 5'UTR (Bellen et al., 2004). Unlike *Tik* or *TikR*, *CK2*^{MB00477} is pupal lethal when homozygous, suggesting that this allele is a hypomorph. *CK2*^{MB00477} does not elicit any eye or bristle defects in the heterozygous state.

Phosphorylation and spatial regulation of M8 activity during eye development:

As mentioned above, emerging evidence suggests that phosphorylation of M8 by CK2 is at the heart of lateral inhibition. Specifically, it was observed that CK2 phosphorylation augments repression by M8. Ectopic expression of CK2 phosphomimetic (M8SD) variant elicits a severely reduced eye, akin to *E(spl)D*/M8* (see above). To account for these apparently similar behaviors of M8* and M8SD, an autoinhibition model (see above) was proposed. Based on this model, phosphorylation by CK2 relieves the CtD mediated intramolecular inhibition and, consequently, converts M8 into an active repressor (Fig. 7).

In line with the autoinhibition model, one would expect that both M8* (lacking the CtD), and M8SD (phosphomimetic variant) should function as constitutively active repressors during eye development. This repressor activity should be greater at stage-1 (during proneural enhancement) than at stage-2/3 (lateral inhibition), because Ato levels are lower at the former stage of the MF.

However, contrary to this prediction, the dominant eye defects of both M8* and M8SD have been found to be MF stage specific. While M8* elicits a severe reduced eye

when expressed at stage 1 (proneural enhancement), it is virtually ineffective at stage 2/3 (lateral inhibition stage). These observations suggest that, once the Ato 'self-stimulatory' loop is established, and Ato levels rise, M8* loses its effects (discussed in chapter-2). In contrast, the dominant eye defects of M8SD are stage 2/3 specific where lateral inhibition refines R8's from preclusters. Why do these two protein variants display non-concordance with respect to their stage-specificity?

The CK2 motif in M8 resides downstream of a region, rich in Ser-residues (Fig. 9). Interestingly, these residues are conserved in Drosophila M5/7, and also in murine and human Hes6 (Fig. 9). The clustering raises the possibility of secondary (hierarchical) phosphorylation by other protein kinases. Additionally, secondary phosphorylation (CK2 and MAPK) has been demonstrated to be necessary in the case of Hes6 activity (Belanger-Jasmin et al., 2007). If a similar situation were to apply to M8 as well, multisite phosphorylation could be essential for repression by M8. Based on the observations, that M8SD only elicits eye defects at stage 2/3, but not at stage 1, the possibility arises that multisite phosphorylation is likely to occur at stage 2/3 (discussed in chapter-2), and these modifications then drive repression.

Further analysis of the P-domain reveals that apart from Ser^{159}, three other Ser residues are conserved in M8, $Ser^{151, 154, 155}$. Of them Ser^{151} meets the consensus (PxSP) for MAPK. Furthermore, pSer at Ser^{159} (CK2 site) generates a consensus for GSK3 at Ser^{155}, and pSer at Ser^{151} (MAPK), generates a consensus for CK1 at Ser^{154}, therefore raising the possibility of hierarchical phosphorylation. Although, no R8 defects have been associated with either CK1 or GSK3 mutations, MAPK has for long been thought to play critical roles during R8 specification (see below), although the mechanism was unknown. This is of particular relevance because MAPK's are activated by RTK's, and mutations in EGFR have been known to affect R8 patterning.

The Epidermal Growth Factor Receptor:

The conserved Epidermal Growth Factor Receptor (EGFR) is a member of the RTK familiy, which regulate diverse processes critical for metazoan development, including proliferation, survival, cell-fate specification, and differentiation. Owing to the functional pleiotropy of EGFR, as well as its involvement in human cancer, extensive efforts have been directed to unravel the signal transduction cascade as well as the underlying mechanisms (Yarden, 2001). Coordinated efforts in Drosophila*, C.elegans* and mammalian cell lines have led to a broad understanding of the pathway (Voas and Rebay, 2004; Wassarman et al., 1995). EGFR is represented by a single gene in both Drosophila (*DER*) and *C.elegans* (*let-23*), while in mammals it consists of four members, which include EGFR/ErbB1/HER1, ErbB2/HER2/Neu, ErbB3/HER3 and ErbB4/HER4 (Yarden, 2001).

EGFR's are transmembrane receptors, which contain extracellular ligand binding domain, a single transmembrane helix and an intracellular tyrosine kinase motif. In the absence of the ligands, EGFR is monomeric, and exhibits no kinase activity. Ligand binding activates the receptors, leading to their dimerization. This facilitates 'transphosphorylation' where one receptor monomer phosphorylates the other partner. Once activated, they mediate signals either by phosphorylating downstream targets or by recruiting protein complexes, which recognize specific phosphotyrosine residues (Geer et al., 1994). Given the fact that there is only one EGFR isoform in both Drosophila and *C.elegans*, heterodimerization does not occur in these two organisms. In contrast, the presence of four EGFRs in mammals facilitates both homo-and heterodimerization (Schlessinger, 2000). Receptor heterodimerization of EGFR have been attributed to diversity, specificity of function, signaling level, etc. (Schlessinger, 2000). Diversity of signaling in the case of Drosophila and *C.elegans*, is achieved through variable N-linked glycosylations of the

receptor, by different ligands, or by differential localization or targeting of the receptor (Moghal and Sternberg, 1999).

Ligands of EGFR:

In the case of Drosophila, there are four activating ligands and one inhibitory ligand. Three of the ligands, Spitz (Spi), Keren (Krn), and Gurken (Grk) belong to the TGF-α family of proteins, and are produced as transmembrane precursors (Neuman-Silberberg and Schupbach, 1993; Reich and Shilo, 2002; Rutledge et al., 1992). Spi is the primary Drosophila EGF receptor (DER) ligand, and activates the pathway in most contexts in Drosophila development, and plays a critical role during Drosophila eye development (Freeman, 1994b). Krn and Grk are used much less frequently, and their roles are not fully clear. Another activating ligand, Vein (Vn), belongs to the Neuregulin family, and is utilized in tissues requiring low EGFR signaling (Schnepp et al., 1996; Shilo, 2003). Vn does not play a major role during eye development (Shilo, 2003). The fifth ligand, Argos (Aos) is unique to Drosophila. Aos antagonizes DER activation (Freeman, 1994a; Freeman et al., 1992), and it has been found that DER signaling leads to enhanced *aos* expression. This observation suggests that a negative feedback loop is a critical regulatory step in restricting high signal output (Golembo et al., 1996; Wasserman and Freeman, 1998).

The MAP Kinase Cascade:

Among the different signaling pathways activated by EGFR, the MAP kinase cascade is widely studied. To date, a number of distinct MAP kinases have been identified and include extracellular regulated kinases (Erks1/2), Jun N-terminal kinases (Jnks1/2) , and p38a/b. All of these are Ser/Thr protein kinases and function through a phosphorylation

cascade to ultimately phosphorylate various transcription factors that, in turn, regulate transcription. The process starts with the phosphorylation of a MAPK kinase kinase (MAPKKK), which in turn phosphorylates MAP kinase (MAPKK). Activated MAPKK then phosphoryaltes MAPK, which then phosphorylates protein targets in both the cytoplasm and the nucleus (Reiser et al., 1999).

EGFR signaling in Drosophila:

The most commonly used EGFR pathway in the Drosophila is the Ras/Raf/MAPK pathway. The pathway initiates with Spi binding to the extracellular domain of DER, leading to the activation of a GTPase called Ras (Seger and Krebs, 1995). Activated Ras then activates Raf1 (MAPKKK) (Chong et al., 2003; Seger and Krebs, 1995), which then phosphorylates and activates the dual specificity kinase MEK (MAPKK). The process culminates with the MEK-dependent phosphorylation of ERK (MAPK) at Tyr and Thr residues within the activation loop (Zhang et al., 1995). Dual-phosphorylated MAPK (dpERK) then phosphorylates its targets in both the cytoplasm and the nucleus (Reiser et al., 1999). In Drosophila, dpMAPK phosphorylates specific target proteins in the nucleus, including Pointed (Pnt) and Yan (Aop) (O'Neill et al., 1994), homologs of human Ets-1 and Tel-1, respectively (Golub et al., 1994; Watson et al., 1985). Both the proteins harbor an Ets-DNA binding domain, by virtue of which they compete for the regulatory regions of downstream transcriptional targets. In the absence of dpERK, Yan represses target genes (Golub et al., 1994), while Pnt functions as a transcriptional activator only upon phosphorylation by dpERK (Watson et al., 1985).

Spi-mediated activation of EGFR also upregulates transcription of the inhibitory ligand Aos (Golembo et al., 1996). Although early studies suggested that Aos antagonizes DER through direct interactions with the receptor (Jin et al., 2000; Vinós and Freeman,

2000), recent studies suggest that Aos antagonizes EGFR signaling by directly binding to Spi itself (Klein et al., 2004). Binding of Aos to Spi prevents the binding of the latter to the DER receptor, and as such Aos helps to maintain a low steady state level of DER signaling in a spatial and temporal manner (Klein et al., 2004).

Eye development and DER signaling in Drosophila:

DER signaling is reiteratively employed at different stages of fly eye development. From the specification of the eye field to the specification of different photoreceptors (R-cells), DER works either co-operatively or antagonistically with Notch.

As discussed above, the eye develops from a monolayer primordium, known as the eye disc. Differentiation of the eye disc initiates at the posterior of the disc with the formation and movement of the MF. The MF progresses from the posterior of the eye disc to the anterior. Cells anterior of the furrow are mitotically active, whereas those posterior to it undergo specification and differentiation. The initiation of the MF is the outcome of the coordinated interplay of several signaling pathways (Heberlein et al., 1998). DER plays critical roles during initiation of the MF, and acts upstream of Hh and Dpp (Kumar and Moses, 2001). In this context, DER operates cooperatively with N to potentiate MF initiation. Studies involving conditional mutants and tissue and temporal specific overexpression of activated components of DER pathway have identified the importance of DER. It is required for initiation of the MF (birth), and also for formation of the MF along the lateral margins (MF-reincarnation) of the eye disc (Kumar and Moses, 2001). Studies conducted by Kumar and Moses suggested that DER acts upstream of both Notch and Hh during MF initiation, and during its reincarnation (Kumar and Moses, 2001). DER mediated expression of Hh induces differentiation of photoreceptors, which in turn lead to additional DER signaling and

the establishment of a positive feedback loop. The positive feedback loop facilitates continued forward progression of the MF (Rogers et al., 2005).

DER signaling during R8 specification in Drosophila:

A number of different and contradictory views exist for the potential roles of DER in R8 specification. It was initially proposed that DER is involved in R8 specification based on the observation that the gain of function DER allele, *Ellipse (Elp)* affects R8 selection from preclusters, resulting in loss of R8 photoreceptors (Baker and Rubin, 1989). Furthermore, *Elp* exacerbates the retinal defects of *Notch* allele, N^{spl} (Baker and Rubin, 1992). As mentioned above, N^{spl} is a gain of function allele that renders the R8 precursors sensitive to inhibitory Notch signaling. Based on these observations, it has been proposed that DER activity is required for prompt execution of lateral inhibition. The fact that Ras/MAPK is active in the intermediate groups and in the cells immediately posterior is supported by elevated levels of dpMAPK in the intermediate groups in wild-type eye discs (Baonza et al., 2001; Kumar et al., 1998; Lesokhin et al., 1999; Rodrigues et al., 2005; Yang and Baker, 2001). This dpMAPK staining is absent in DER^{null} clones, suggesting that activated MAPK is dependent on DER signaling. Based on these observations it has been suggested that DER may play a parallel role along with Notch in lateral inhibition to restrict R8 fate to a single cell within the proneural clusters (Baker and Rubin, 1989; Dokucu et al., 1996; Dominguez et al., 1998; Lesokhin et al., 1999; Yang and Baker, 2001).

This suggestion is challenged by other studies on DER^{null} clones. DER^{null} clones generated in a *Minute* background do not elicit any R8 specification defects, suggesting that DER is not essential for R8 specification (Baonza et al., 2001; Dominguez et al., 1998; Lesokhin et al., 1999; Yang and Baker, 2001). The dispensability of DER function during R8 specification was further underscored by the observation that R8 specification was

unperturbed in flies carrying the temperature-sensitive *DER*^{tsla} allele, reared at the restrictive temperature.

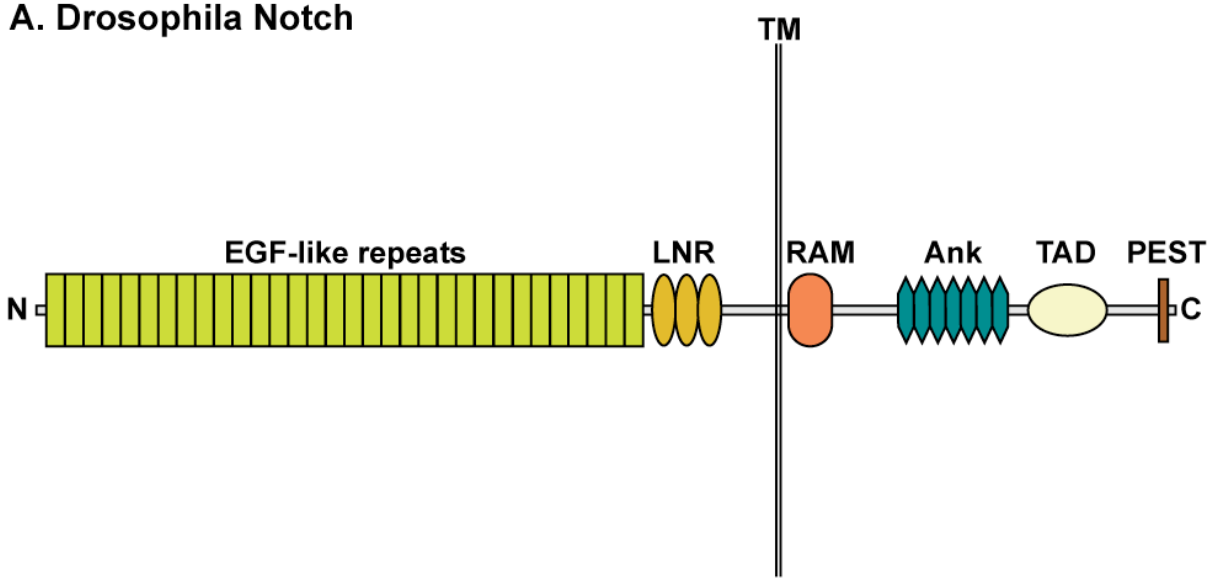
However, the observation that *DER*^{null} and *Ras* mutant clones in *Minute* background elicit aberrant and perturbed R8 spacing raised the potential role of DER in R8 spacing (Baonza et al., 2001; Dominguez et al., 1998; Lesokhin et al., 1999; Yang and Baker, 2001). But this claim is challenged by the observation that *Minute*$^{+}$ clones could elicit non-cell autonomous effects on Ato expression, and consequently, R8 spacing (Rodrigues et al., 2005). Another intriguing observation is the behavior of the *DER*^{tsla} mutant clones that initially maintain proper R8 spacing, but later elicit R8 spacing defects. This observation led to the argument that DER is involved in maintenance, rather than initiation of R8 spacing (Kumar et al., 1998; Rodrigues et al., 2005).

Although DER is indeed active in intermediate groups, the precise role of DER in R8 founder cell specification remains enigmatic. The studies described in chapter-4 attempt to answer this conundrum. These studies suggest that both DER and Notch act cooperatively at the level of M8 during lateral inhibition to specify single R8's within the proneural clusters. These multiple phosphorylation events may function as multiple checkpoints during R8 specifications, which prevent a precocious onset of repression. The absence of CtD in M8* (*E(spl)D*) bypasses these regulatory steps, therefore, leading to a conformation that is predisposed to binding to Ato.

The studies described in this dissertation seek to address several aspects of repression by M8 during R8 selection and patterning. These studies provide a fundamental reinterpretation of the mechanism of the classic *E(spl)* allele*, E(spl)D* (chapter-2, Kahali et al., 2009), provide *in vivo* evidence of the auto-inhibition model (chapter-3, Kahali et al., 2010), implicate the importance of secondary phosphorylation in regulating M8 activity (chapter-4), demonstrate direct genetic interactions between alleles of *CK2, E(spl)* and

Notch (chapter-5), and conclude with the identification and characterization of bHLH Hairy, a member of the HES family, as a target of CK2 (chapter-6, Kahali et al., 2008).

A. Drosophila Notch



B. Notch pathway components

Component Function	Drosophila	C. elegans	Mammals
Receptor	Notch	LIN-12	Notch-1, -2, -3, -4
Ligand	Delta, Serrate	LAG-2	Delta-1, -3, -4 Jagged-1, -2
Nuclear Effectors	Su(H)	LAG-1	RBPjκ/CBF-1
Canonical Target	E(spl)	REF-1	Hes/Hey

Figure 1. Drosophila Notch receptor and core components. (A) Schematic representation of the Drosophila Notch receptor. (B) Core components of Notch pathway in different species.

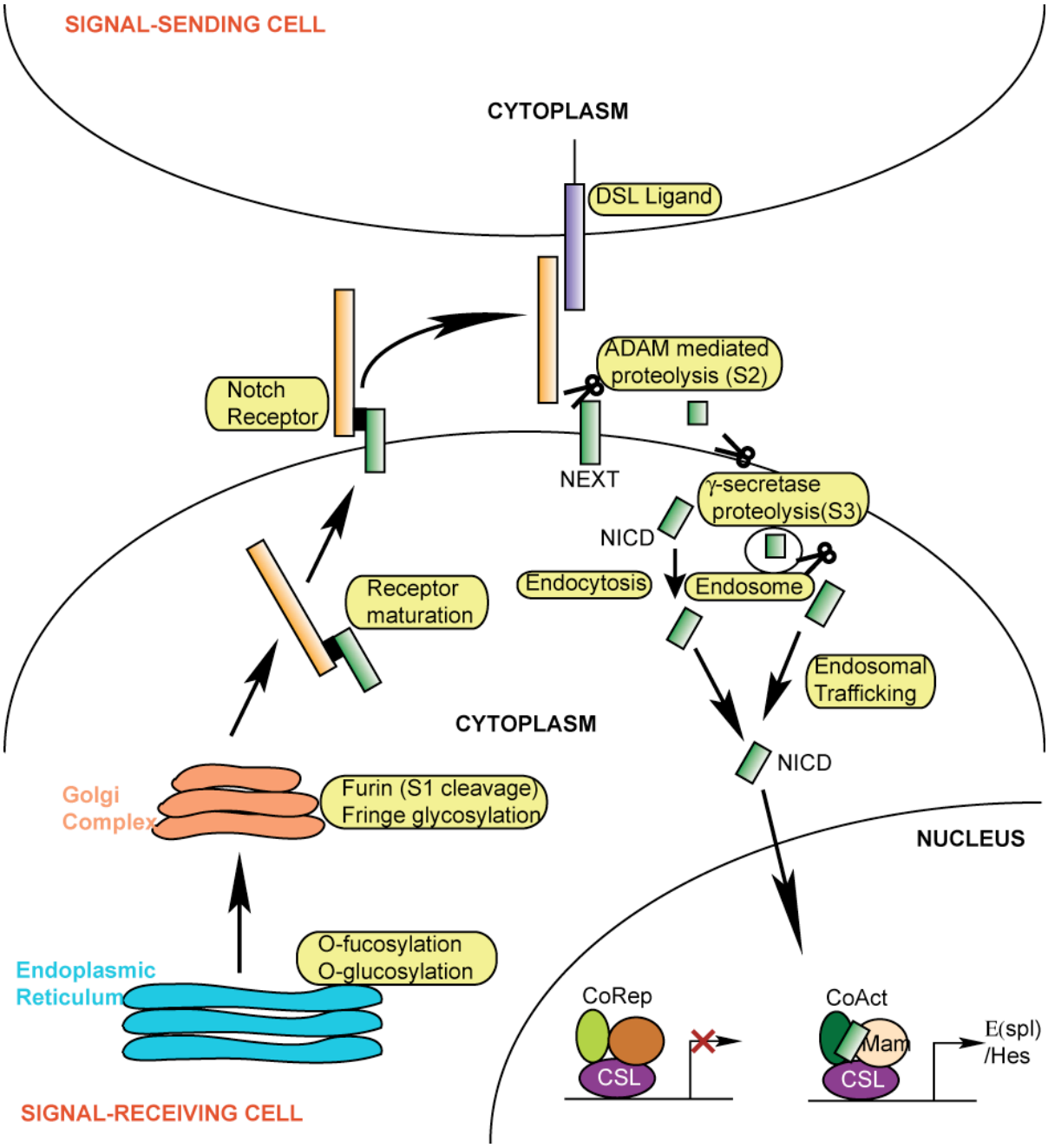


Figure 2. Regulated proteolysis in Notch receptor processing. Glycosylation and cleavage by Furin (S1 cleavage) resulted in the mature receptor. Upon ligand binding, Notch undergoes two consecutive proteolytic cleavages to release the NICD, which translocates to the nucleus and mediates transcription of the *E(spl)/HES* genes.

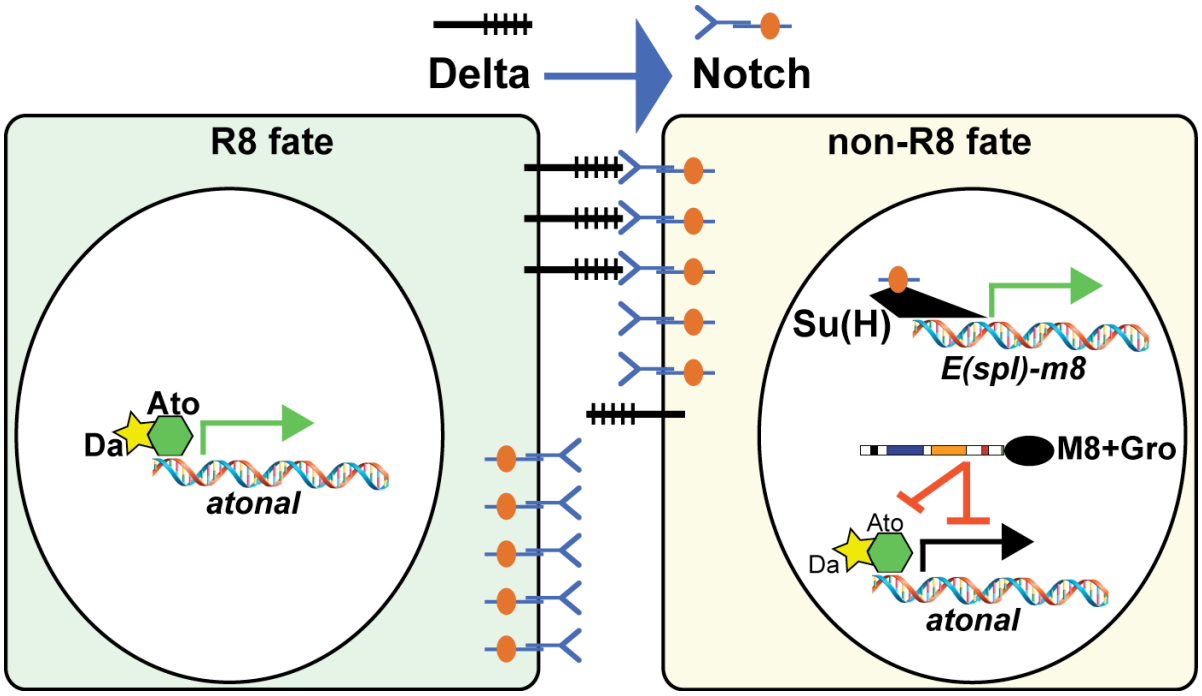


Figure 3. Notch mediated lateral inhibition during Drosophila R8 specification The future R8 cell expresses higher amount of Notch ligand Delta, which binds to the Notch receptor in the non-R8 cell, and triggers inhibitory Notch signaling. The pathway culminates with the expression of E(spl) repressors (M8 is shown) that complexes with Groucho and antagonizes the proneural protein Atonal.

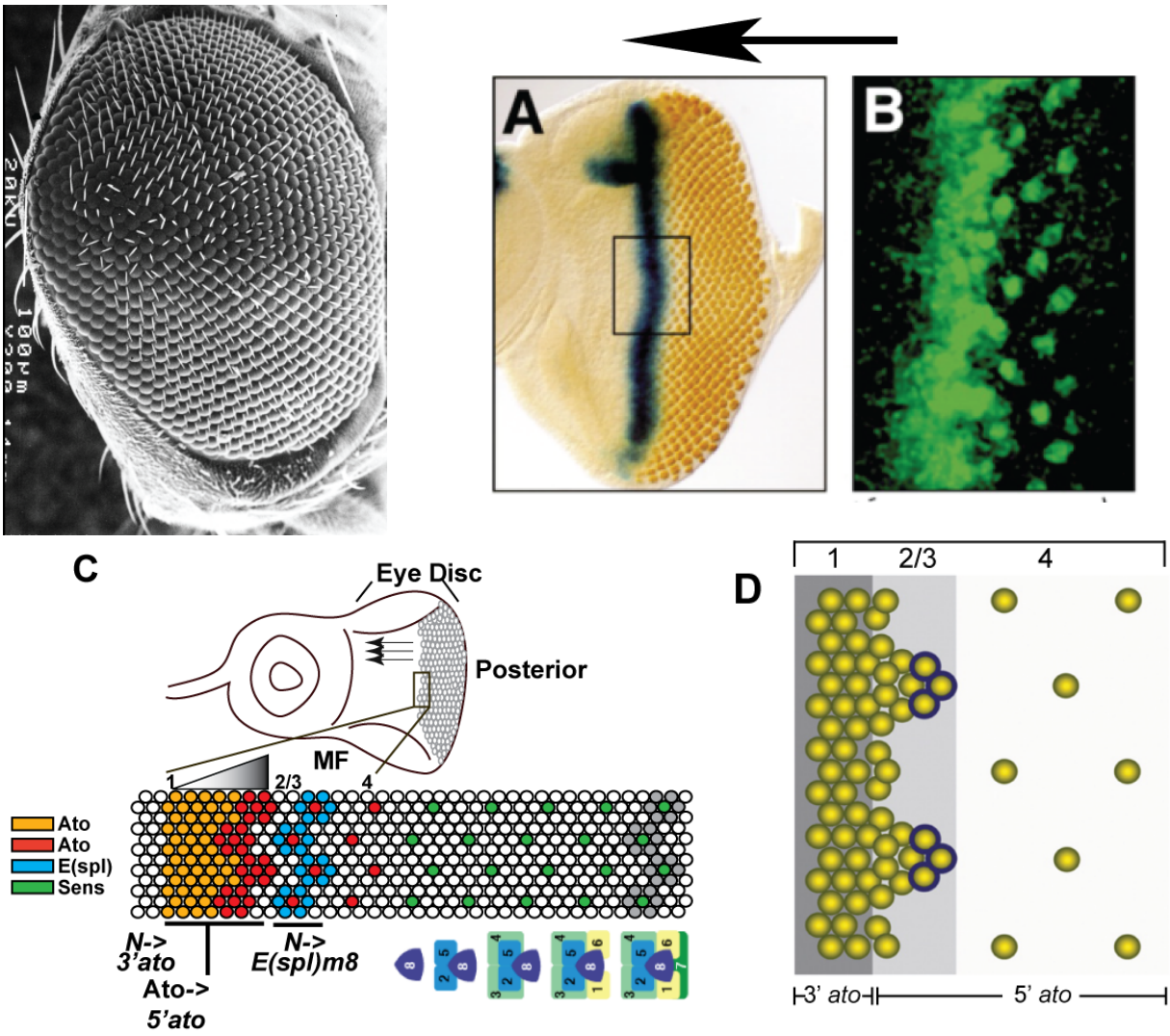


Figure 4. R8 specification and retinal patterning of the Drosophila eye. (A) Third instar larval eye disc showing the morphogenetic furrow (MF, blue stripe). Arrow showing the progression of the MF. (B) Displaying the expression pattern of the proneural protein Atonal (Ato) in the eye disc. Ato is expressed ubiquitously anterior of the MF, but resolve into single cell posteriorly (R8 precursors). (C) Biphasic role of Notch during R8 specification. (D) Schematic of Ato expression during R8 specification (adapted from Frankfort and Mardon, 2002).

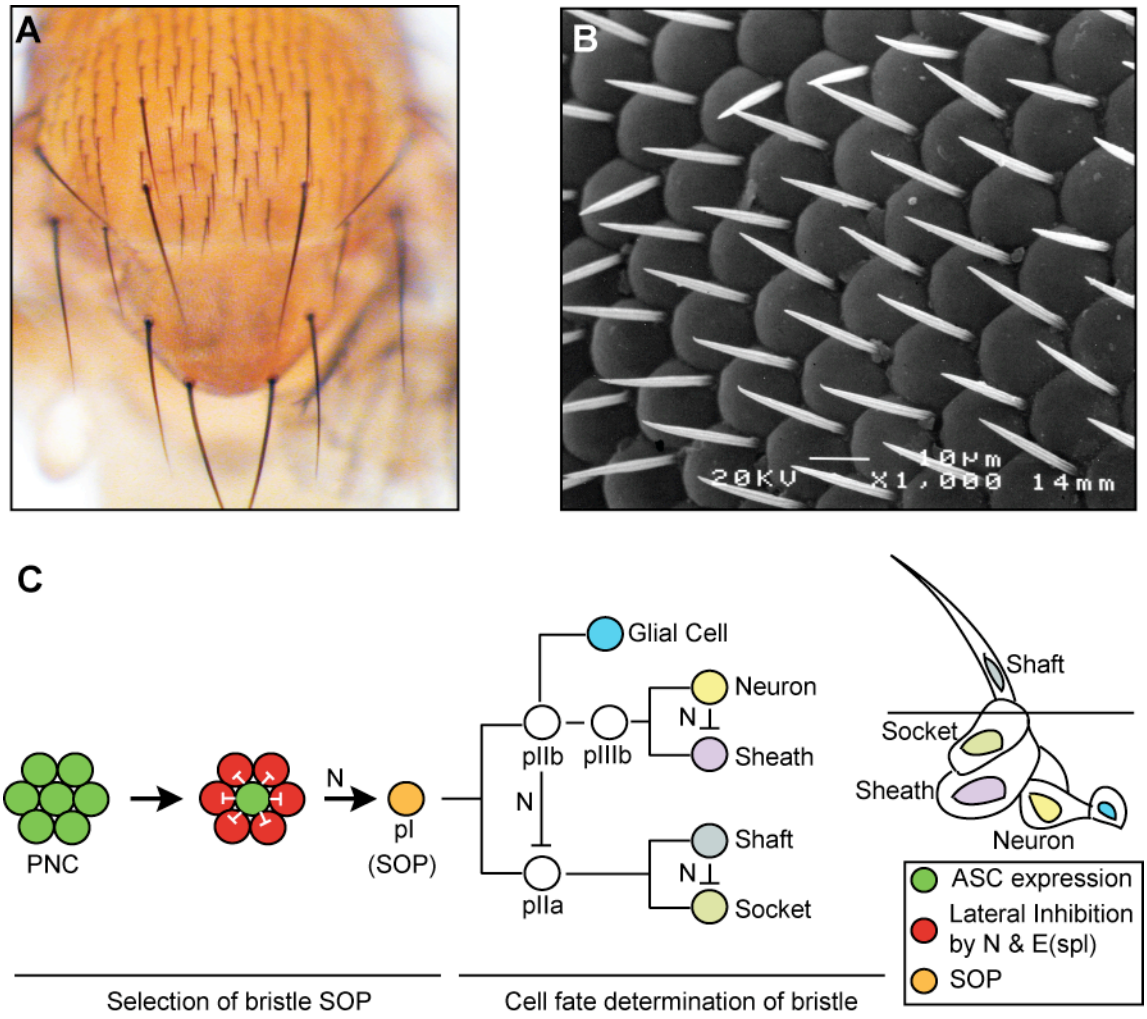


Figure 5. Patterning and morphogenesis of the Drosophila bristle. (A) The position of the Macrochaetes (MC's) and microchaetes (mc's) on the notal region and the interommatidial bristles (IOB's, B). (C) Notch mediated lateral inhibition during bristle SOP selection, and the asymmetric divisions of the SOP.

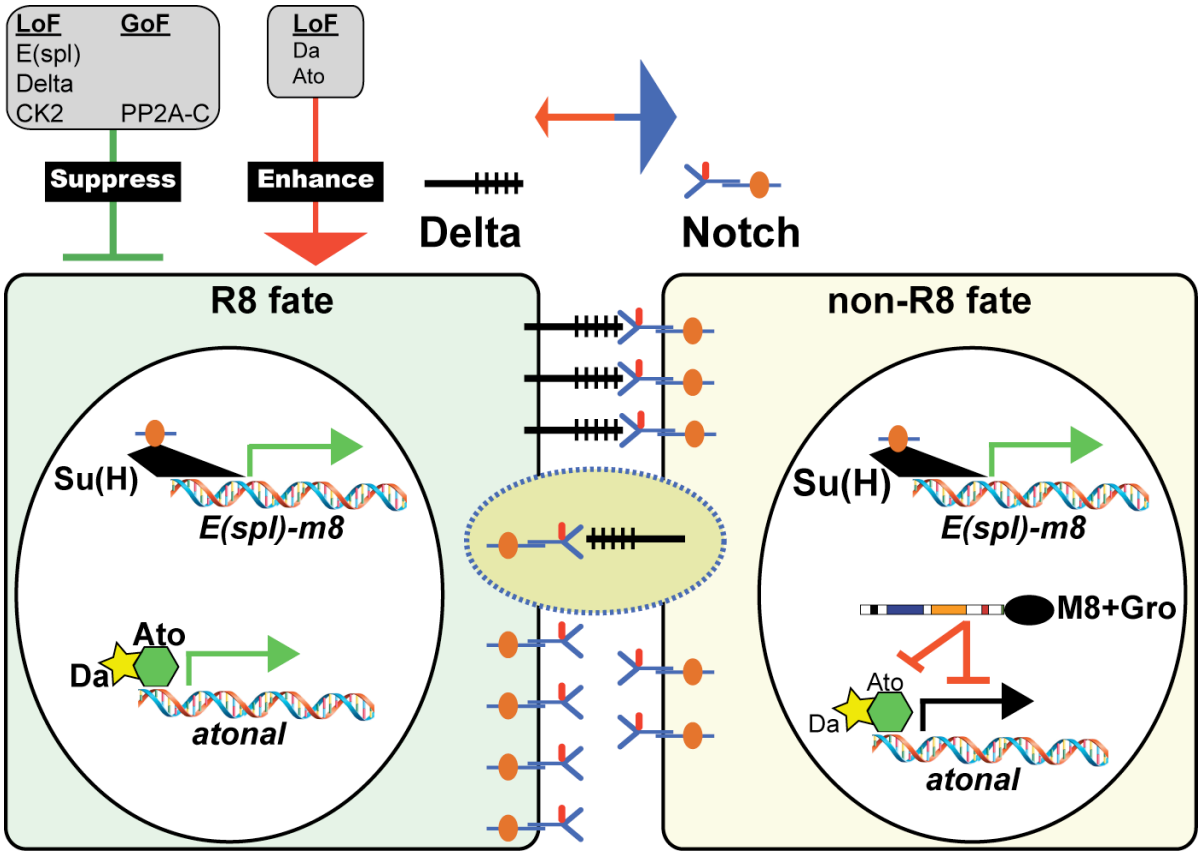


Figure 6. Altered Notch signaling by the N^{spl} mutation. In N^{spl} allele, substitution of $Ile^{578}Thr$ in EGF-repeats 14 leads to a mis-glycosylation, resulting in receptor with greater signaling strength. Consequently, N^{spl} receptor exhibits increased interaction with ligand Delta (Dl). As a result, some of the presumptive R8's became susceptible to inhibitory Notch signaling, resulting in loss of eye field.

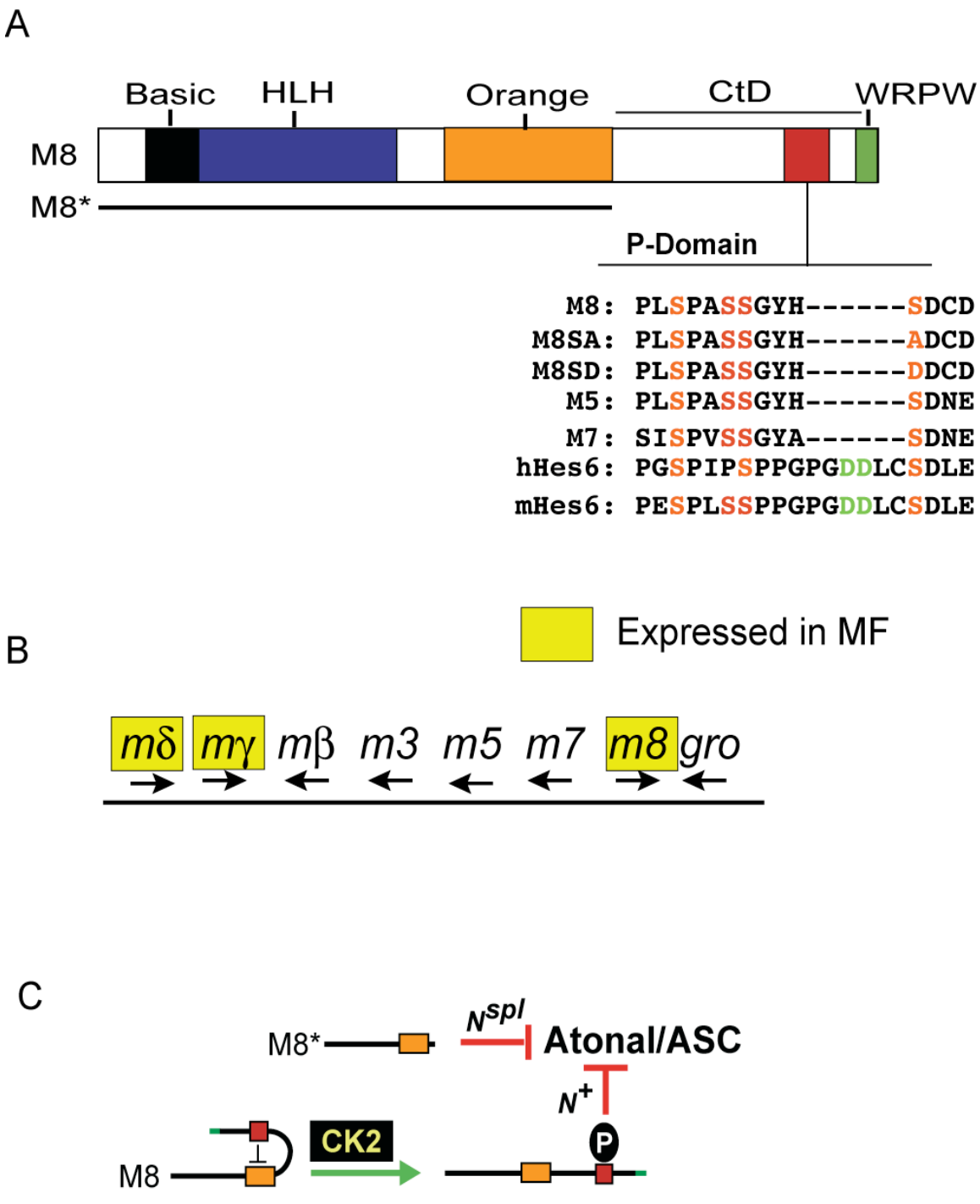
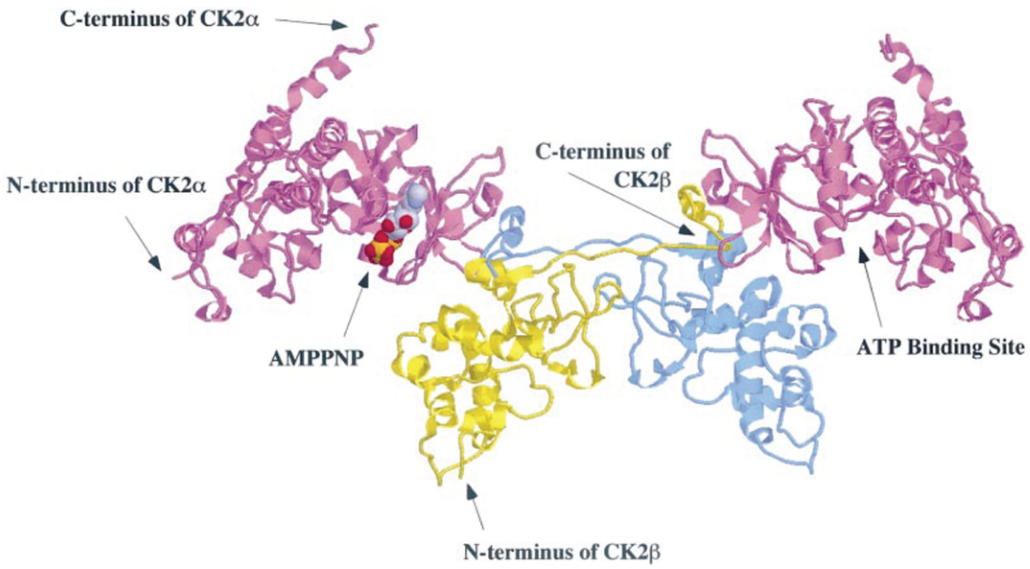


Figure 7. Structure of M8 and regulation of repression by autoinhibition. (A) Domain organization of M8, showing the conserved phosphorylation motif (P-Domain). (B)

Schematic representations of genomic organization of *E(spl)* locus. (C) Autoinhibition model of M8 regulation.

Structure of CK2 holoenzyme



Alleles of the α (catalytic) subunit of CK2

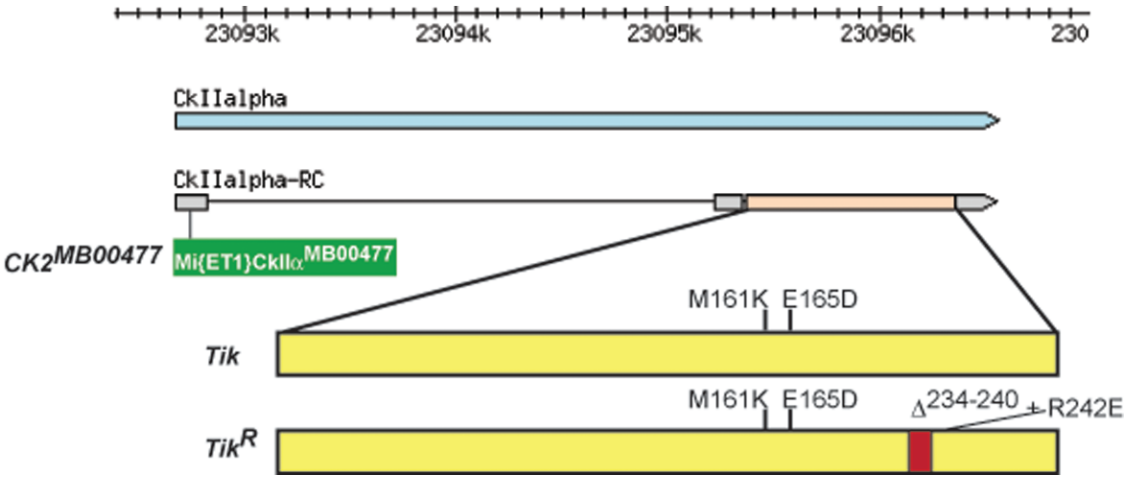


Figure 8. Structure of CK2 holoenzyme and alleles of Drosophila *CK2α*. The ribbon diagram of CK2 holoenzyme (Niefind et al., 2001), displaying the assembly of catalytic (α) sununits (magenta) on a CK2β dimer (yellow and blue). The alleles of CK2α and their corresponding lesions are indicated. Note that in *CK2*^{MB00477} the *minos* insertion affects the 5' UTR, whereas *Tik* and *TikR* are mutations affecting the protein structure.

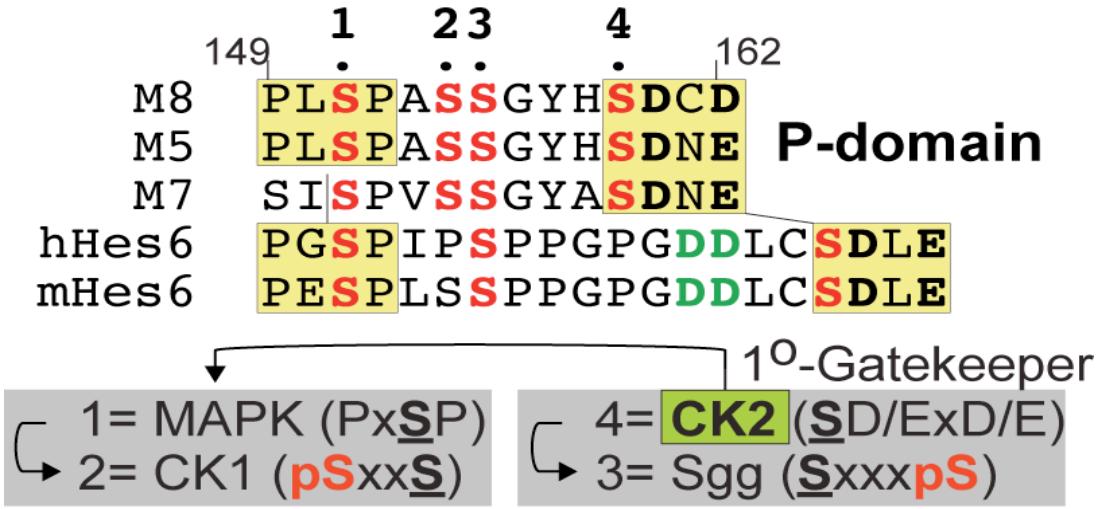


Figure 9. Phosphorylation domain of M8 and related Hes proteins. Conserved phosphorylation sites in Drosophila M8/5/7, human (h), and murine (m) Hes6. Ser^{151} and Ser^{159} meet the consensus for MAPK and CK2 respectively. Ser^{154} and Ser^{155} meet the consensus for CK1 and Gsk3 (Sgg) respectively. However, the phosphorylation by CK1 and Sgg depends on the phosphorylation status of Ser^{151} and Ser^{159} respectively. Phosphorylation by CK1 and Sgg may be dispensable in case of mammals, owing to the presence of two Asp residues in the intervening sequences.

CHAPTER 2

On the mechanism underlying the divergent retinal and bristle defects of M8* (*E(spl)D*) in Drosophila.

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Abstract:

Our results, using endogenous mutants and Gal4-*UAS* driven transgenes, implicate multisite phosphorylation in repression by E(spl)M8. We propose that these phosphorylations occur in the morphogenetic furrow (MF) to reverse an auto-inhibited state of M8, enabling repression of Atonal during R8 specification. Our studies address the paradoxical behavior of M8*, the truncated protein encoded by *E(spl)D*. We suggest that differences in N signaling in the bristle versus the eye underlie the antimorphic activity of M8* in N^{+} (ectopic bristles) and hypermorphic activity in N^{spl} (reduced eye). Ectopic M8* impairs eye development (in N^{spl}) only during establishment of the *atonal* feedback loop (anterior to the MF), but is ineffective after this time point. In contrast, a CK2 phosphomimetic M8 lacking Groucho (Gro) binding, M8SDΔGro, acts antimorphic in N^{+} and suppresses the eye/R8 and bristle defects of N^{spl}, as does reduced dosage of *E(spl)* or CK2. Multisite phosphorylation could serve as a checkpoint to enable a precise onset of repression, and this is bypassed in M8*. Additional implications are discussed.

Introduction:

The Drosophila sensory organs (eye and bristles) have been widely studied as models for neuronal specification and have provided important insights into the mechanisms underlying neurogenesis. The onset of neural cell fate specification in these sense organs involves expression of the basic-helix-loop-helix (bHLH) transcriptional activators encoded by the *achaete-scute Complex* (*ASC*) and *atonal* (*ato*), which are essential for formation of groups of equipotent cells, the proneural clusters (PNC's). Specification of the bristle-PNC's requires the ASC activators, whereas those that give rise to the 'founding' R8 photoreceptors require Ato (Ghysen and Richelle, 1979; Jarman et al., 1994; Modolell and Campuzano, 1998; Skeath and Carroll, 1991). In either case, however, only a single cell from each PNC goes on to form the bristle sensory organ precursor (SOP) or the R8 cell, a process that is vital for stereotyped patterning of these sensory organs (reviewed in Baker, 2002; Bertrand et al., 2002; Frankfort and Mardon, 2002; Gomez-Skarmeta et al., 2003).

R8/SOP selection involves inhibitory Notch (N) signaling between the future R8/SOP and its immediate neighbors in a PNC (reviewed in Artavanis-Tsakonas et al., 1999; Bray, 2006; Mumm and Kopan, 2000). This signaling involves interaction of the ligand Delta (Dl) that is expressed at the highest levels in the future R8/SOP, and the N receptor in adjacent cells (Parks et al., 1995; Simpson et al., 1992). As a result, N is cleaved to release its intracellular domain (N^{icd}). This fragment translocates to the nucleus and along with Suppressor of Hairless (Su(H)) elicits transcription of the bHLH repressors encoded by the *Enhancer of split Complex*, *E(spl)C* (Bailey and Posakony, 1995; de Celis et al., 1996; de la Concha et al., 1988; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). The E(spl) repressors in a complex with Gro (Paroush et al., 1994) then antagonize Ato/ASC to redirect cell fate away from the (default) R8/SOP fate. This process, termed 'lateral inhibition'

(Baonza and Freeman, 2001; Lehmann et al., 1983; Simpson, 1990), thus ensures the emergence of a single R8/SOP from each PNC.

Given the importance of E(spl) for lateral inhibition, numerous studies have identified functional domains and their roles in repression. These are: basic (DNA binding), HLH (E(spl) dimerization), Orange (interactions with ASC/Ato) and a C-terminal WRPW tetrapeptide (Gro-binding). Structure function analysis suggests that loss of HLH, Orange or WRPW domains, individually, render E(spl) proteins non-functional (Giebel and Campos-Ortega, 1997). The role of WRPW is, perhaps, the best understood, since Gro is an essential co-repressor for all E(spl) members (Fisher et al., 1996; Paroush et al., 1994). One exception, however, is *E(spl)D*, a unique dominant mutant allele of the *E(spl)* member *m8* that abrogates eye development in the presence of the *split* allele of *N*, i.e., N^{spl}.

E(spl)D harbors two mutations; one stabilizes the *m8* transcript, and the other truncates the protein after the Orange domain. This truncated protein called M8* lacks 56 C-terminal residues including WRPW, and thus cannot bind Gro (Nagel et al., 1999; Tietze et al., 1992). Importantly, over-expression of a *UAS-m8** construct during eye development mimics the effects of *E(spl)D*, indicating that the truncation in M8* underlies its enhancement of N^{spl}. It has, therefore, been suggested that *E(spl)D* is a *gro*-independent hypermorph (Nagel et al., 1999). However, in N^{+} flies, M8* acts as an antimorph and elicits ectopic bristles, an unexpected finding since removal of the C-terminal tetrapeptide WRPW *per se* from full-length M8 renders the protein non-functional (Giebel and Campos-Ortega, 1997). The mechanism underlying these divergent activities of M8* in N^{+}-vs-N^{spl} remained unresolved.

Studies conducted by our laboratory have demonstrated that protein kinase CK2 phosphorylates M8 at Ser^{159} in the C-terminal domain (CtD, Trott et al., 2001) that is missing in M8*. The role of this modification was suggested by the finding that the CK2

'phosphomimetic' variant (M8S^{159}D) blocked R8 specification and eye development, and mimicked the effects of M8*, albeit in N^{+} flies (Karandikar et al., 2004). No such effects were seen with wild type M8. The observation that M8* and M8SD, but not M8, interact strongly with Ato (in yeast), raised the possibility that the non-phosphorylated CtD 'autoinhibits' Orange to restrain repression by M8 (Karandikar et al., 2004, see Fig. 1b). In the case of M8, phosphorylation would displace the CtD to expose Orange and enable repression, and the absence of this autoinhibitory region in M8* might thus enable it to precociously bind and antagonize Ato.

Given the similar eye phenotypes elicited by M8SD and M8*, we hypothesized that M8SD might provide a means to uncover the mechanism underlying the divergent activities of M8* in N^{+}-vs-N^{spl}, and further define the regulatory influence of phosphorylation on repression by E(spl) in vivo. Since M8* is unable to bind Gro, we sought to assess the role of WRPW in the retinal defects of M8SD, with the expectation that eliminating the ability of M8SD to bind Gro might render it N^{spl}-dependent, akin to M8*. We describe here the unexpected antimorphic activity of this variant, which we call M8SDΔGro. In N^{+} flies, M8SDΔGro elicits supernumerary (ectopic) bristles and potently suppresses the bristle loss phenotype of ectopic M8, but does not interfere with R8 specification or eye development, effects similar to those reported for M8* (Giebel and Campos-Ortega, 1997; Nagel et al., 1999). However, M8SDΔGro and M8* behave very differently in the N^{spl} background. While M8* exacerbates the reduced eye of N^{spl}, M8SDΔGro suppresses the eye defects, especially the specification of the R8 cells. This suppression is specific to the time and place where R8 photoreceptors are specified, i.e., the morphogenetic furrow (MF). The possibility that the antimorphic activity of M8SDΔGro mediates its effects in N^{+} or in N^{spl} is supported by our findings that $Df(3R)^{BX22}$, a deficiency that uncovers *m5*, *m7*, *m8* and *gro*, also suppresses N^{spl}, as does knockdown of CK2. No antimorphic behavior in N^{+}, or suppression of N^{spl} is

seen with the Gro-neutralized variant of M8 or with its non-phosphorylatable form M8S^{159}A. Our studies suggest that differences in N signaling between the bristle and the eye, rather than different mechanisms, underlie the antimorphic and hypermorphic activities of M8* in N^{+} and N^{spl}, respectively.

Results:

The eye/R8 defects of M8SD are rescued by increased dosage of Ato.

The retinal defects of M8SD mimicked those of M8*, albeit in N^{+} flies. Since the effects of *E(spl)D* (M8*) are sensitive to Ato dosage (Nagel et al., 1999), we first tested if this is the case for M8SD. Expression of M8SD with *scaGal4* elicits a severely reduced eye (~ 15 ommatidia, Karandikar et al., 2004), and the possibility remained that this is a limit phenotype, i.e., beyond a threshold and not amenable to modulation. We therefore used the weaker driver *109-68Gal4* (active in PNC's and in R8's, Powell et al., 2004). Expression of M8SD with *109-68Gal4* elicits a moderately reduced eye at 24^{o}C (Fig. 1c), and eye discs display sporadic and inconsistent levels of Senseless (Sens) along the DV or AP axis (Fig. 1c'). Furthermore, ommatidial size varied upon expression of M8SD (Fig. 1c) and staining of discs with Sens+ELAV showed photoreceptor clusters with reduced numbers of secondary R-cells (data not shown). Since Sens is essential for R8 differentiation (Frankfort et al., 2001; Nolo et al., 2000), the possibility arises that the remaining R8's are likely to be functionally compromised, and might not recruit secondary photoreceptors normally. Both, the eye and R8 defects of M8SD were suppressed by co-expression of a *UAS-ato* construct (Fig. 1e, e'), suggesting that Ato is an in vivo target of M8SD (as is the case for M8*). Expression of *UAS-ato*, by itself, does not elicit overt eye/R8 defects at 24^{o}C (Fig. 1d, d'), reflecting the observation that *109-68Gal4/+; UAS-ato/+* flies display rough eyes and supernumerary R8's in a more pronounced manner only at 29^{o}C (White and Jarman, 2000).

Loss of Gro-binding abolishes the reduced eye of M8SD, but not its bristle defects.

To remain consistent with previous studies, we abolished Gro-binding by removal of the WRPW motif (Paroush et al., 1994). Henceforth, we refer to these variants as M8ΔGro, M8SAΔGro and M8SDΔGro (Fig. 2a). In contrast to the full-length proteins, these variants do not interact with Gro, as is also the case with M8* (Fig. 2b). For in vivo analysis, we generated multiple independent insertions of UAS-constructs encoding all three constructs (Fig. 2a); the single insertion of *UAS-m8** has been previously used by others and us (Karandikar et al., 2004; Nagel et al., 1999).

As previously described (Karandikar et al., 2004), expression of M8SD with *scaGal4* elicited a severely reduced eye and loss of the scutellar macrochaetes (MC's) and interommatidial bristles (IOB's), whereas wild type M8 only elicits loss of the MC's and IOB's (Fig. 2c, g, g'). Expression of M8* did not elicit a reduced eye, as these studies were conducted in N^{+} flies. To a large extent, these in vivo effects of M8SD and M8 were neutralized by removal of WRPW (Fig. 2c). However, M8SDΔGro, in particular, was not rendered non-functional. Its expression with *scaGal4* elicited ectopic MC's in 73% flies, a penetrance that was significantly higher than the baseline bristle defects of *scaGal4/+* flies (19%, Fig. 2c). Importantly, this phenotype was not intrinsic to any of the 14 independent *UAS-m8SDΔGro* insertions, but was observed only upon their expression with *scaGal4* (data not shown). Expression of M8* also elicited ectopic MC's with a similarly enhanced penetrance (~81%, Fig. 2c). A virtually identical phenotype has been reported by Giebel and Campos-Ortega (1997) using *scaGal4*, and interpreted as dominant-negative (antimorphic) effects. Expression of M8ΔGro or M8SAΔGro was without effect in multiple (14 and 6, respectively) independent lines; in either case ~15-20% flies displayed ectopic MC's, numbers that were indistinguishable from the baseline defects in *scaGal4/+* flies alone (Fig. 2c, and data not shown). Thus, the elicitation of ectopic MC's requires removal of the

entire CtD (M8*), or removal of WRPW in conjunction with a phosphomimetic Asp in place of Ser^{159} (M8SDΔGro).

M8SDΔGro mimics the ectopic MC phenotype of M8*.

To independently evaluate the ectopic MC's of M8SDΔGro, we employed the Gal4-driver *G455.2*, since its intrinsic MC defects are lower than those of *scaGal4* and because its expression is restricted to the scutellum (Giebel and Campos-Ortega, 1997). In the case of *G455.2/+* flies, ~11% display ectopic MC's, and this number was used as the baseline for comparisons (Fig. 2d). As with *scaGal4* (see above), *G455.2* mediated expression of M8SDΔGro led to ectopic MC's in 67% of the flies, and a similar number (74%) was seen upon expression of M8* (Fig. 2d). Once again, expression of M8ΔGro or M8SAΔGro was without effect, as their ectopic MC phenotypes were indistinguishable from the baseline MC defects in *G455.2/+* flies (Fig. 2d). The non-functional behavior of M8ΔGro has been previously reported (Giebel and Campos-Ortega, 1997). No MC defects (ectopic or missing) were intrinsic to multiple independent insertions of *UAS-m8SDΔGro* (14 lines), *UAS-m8ΔGro* (14 lines), *UAS-m8SAΔGro* (6 lines), or *UAS-m8** (1 line), by themselves (data not shown). Thus, expression of M8SDΔGro or M8* was required for eliciting the ectopic MC defects. Moreover, ectopic MC's were elicited with similar penetrance upon expression of all 14 *UAS-m8SDΔGro*, but none of the *UAS-m8ΔGro* or *UAS-m8SAΔGro* insertions (data not shown). As controls, we tested full-length M8 or M8SD (6 *UAS*-lines each), and found that both elicited potent loss of MC's (Fig. 2e).

M8SDΔGro exhibits antimorphic properties.

If M8SDΔGro is antimorphic, the penetrance of its ectopic MC phenotype should be enhanced by a reduction in *E(spl)* dosage. For these studies, we used $Df(3R)^{BX22}$ (uncovers *m5, m7, m8* and *gro*, Delidakis et al., 1991), which was brought through males to avoid

maternal effects. The baseline bristle defects of *G455.2/+* flies are not significantly modified (enhanced/suppressed) by *Df(3R)^{BX22}* (Fig. 2e). In contrast, expression of M8SDΔGro in *Df(3R)^{BX22}/+* flies elicited ectopic MC's with a modestly higher penetrance than in a background wild type for the *E(spl)C* (82-vs-67%, Fig. 2d, e). Expression of M8ΔGro or M8SAΔGro in *Df(3R)^{BX22}* elicited ectopic MC's whose penetrance was indistinguishable from the baseline numbers in the relevant controls (*G455/+* or *G455/+; Df(3R)^{BX22}/+*), suggesting that both are non-functional (Fig. 2e, and data not shown). The potent loss of MC's with M8 or M8SD was not affected by *Df(3R)^{BX22}* (Fig. 2e), reflecting the suggestion that halved *gro* dosage is not rate limiting for repression by E(spl) (Nagel et al., 1999).

The modest (but reproducible) enhancement by *Df(3R)^{BX22}* of the MC defects of M8SDΔGro, raised the possibility that this reflects the timing of expression with *G455.2.* While *G455.2* is PNC-specific (Giebel and Campos-Ortega, 1997), it is unknown if expression with this driver closely correlates with the onset of lateral inhibition or is delayed. Although this potential delay does not hamper MC loss by full-length (functional) M8 or M8SD (Fig. 2e), the timing of expression would be important for an antimorph of E(spl) proteins, which are thought to function as dimers (Alifragis et al., 1997). If expression with *G455.2* were delayed (even slightly), ectopic M8SDΔGro would be less efficiently incorporated into endogenous E(spl) dimers, thus dampening its activity, leading to an underestimate of its 'strength'.

We thus tested if simultaneous co-expression of M8SDΔGro would attenuate the potent MC loss of *G455.2/+; UAS-m8/+* flies (Fig. 2f). This is, indeed, the case. Importantly, 87% flies co-expressing M8+M8SDΔGro displayed 4 MC's/scutellum, a number similar to that in *G455.2/+* flies (89%, see Fig. 2f). No such suppression was observed upon co-expression of M8ΔGro, a variant that does not exhibit any antimorphic properties (Fig. 2f). Co-expression of a *UAS-LacZ* construct did not attenuate the MC loss of M8 (Fig. 2f), or the

ectopic MC phenotype of M8SDΔGro (data not shown), indicating that suppression by M8SDΔGro does not involve competition by two *UAS*-constructs for a rate-limiting amount of Gal4. Thus, the well established antimorphic behavior (ectopic MC's) of M8* (Giebel and Campos-Ortega, 1997) is only seen with the phosphomimetic variant M8SDΔGro.

Compromising CK2 inhibits repression by ectopic M8.

We have previously tested and found that M8 and M8SD elicit bristle loss with similar potencies (Karandikar et al., 2004). However, the possibility remained that these are limit phenotypes. Given the antimorphic activity of M8SDΔGro, we tested whether compromising CK2 would impair the ability of ectopic M8 to elicit loss of MC's, microchaetes (mc's), and IOB's (Fig. 2g, g'). Indeed, these effects were suppressed by the simultaneous expression of a *UAS-CK2α-RNAi* construct (Fig. 2h, h'), which specifically targets the catalytic (α) subunit of Drosophila CK2 (Bose et al., 2006). Similar results were obtained upon co-expression of *UAS-Tik* (Bose et al., 2006), a dominant-negative construct that encodes a catalytically dead CK2α subunit (data not shown). Thus, the autoinhibition attributed to the CtD and its regulation by (CK2) phosphorylation also appear to influence the interaction of M8 with ASC members.

M8SDΔGro suppresses the retinal defects of N^{spl}.

Due to the similar behavior of M8* and M8SDΔGro during bristle and retinal development in N^{+} flies, we next employed N^{spl}, a gain of function allele that renders R8 precursors' sensitive to inhibitory N signaling (Li et al., 2003). This inappropriate N activity negatively impacts R8 specification and differentiation, and consequently N^{spl}/Y flies display a uniformly rough and a reduced eye (Fig. 3a). As expected, and previously described (Nagel et al., 1999), expression of M8* elicited a severely reduced eye in N^{spl}/Y animals (Fig. 3g). Unexpectedly, however, expression of M8SDΔGro with $h^{H10}Gal4$ suppressed the rough

and reduced eye of N^{spl} and appeared to restore ommatidial phasing (see ventral half of eye, Fig. 3b); similar results were obtained upon expression of all 14 independent *UAS-m8SDΔGro* insertions (data not shown). Expression of M8ΔGro or M8SAΔGro (with h^{H10}*Gal4*) did not enhance or suppress the rough and reduced eye of N^{spl} males in multiple independent lines (Fig. 3h and data not shown).

To further assess these findings, we next tested for the spatial requirements for suppression of N^{spl} by M8SDΔGro, by employing drivers whose expression domains relative to the MF are well known. These are *109-68Gal4* (within the MF) and *gmrGal4* (posterior to the MF and after R8 patterning). We find that expression of M8SDΔGro with *109-68Gal4* also suppressed the rough and reduced eye of N^{spl} (Fig. 3c), whereas its expression with *gmrGal4* was without effect (Fig. 3d). A similar study with *scaGal4* (whose expression also occurs within the MF) was not conducted because *sca* is a modifier of N^{spl} (Brand and Campos-Ortega, 1990). As with h^{H10}*Gal4*, expression of M8ΔGro or M8SAΔGro with *109-68Gal4* was without effect in multiple independent insertions (data not shown, and see below). As added controls, we have confirmed that 1) N^{spl}*/Y; UAS-m8SDΔGro/+*, 2) N^{spl}*/Y;* h^{H10}*Gal4/+* or 3) N^{spl}*/Y; 109-68Gal4/+* flies all display rough and reduced eyes characteristic of N^{spl} males (data not shown, and see below); thus expression of M8SDΔGro was required for suppression.

To quantify the efficiency of rescue, we determined the ommatidial (facet) numbers in ≥15 flies of each of the relevant genotypes as described (Jones et al., 2006). In this analysis, N^{spl} males, which display 323±10 ommatidia, represent the baseline (Fig. 3i). Unlike the severely reduced eye of M8* (≤15 facets), expression of M8SDΔGro significantly increased facet numbers (Fig. 3i). This effect was stronger when this variant was expressed with *109-68Gal4* (566±13 facets) as compared to h^{H10}*Gal4* (521±15 facets). Consistent with the adult eye phenotype, expression of M8SDΔGro with *gmrGal4* resulted in 334±16 facets,

a number virtually identical to that in N^{spl} (Fig. 3i). Thus, M8SDΔGro is ineffective in modulating N^{spl} when expressed posterior to the MF and after R8 specification. Facet numbers did not increase upon expression of M8ΔGro or M8SAΔGro (Fig. 3i), suggesting that these variants are likely nonfunctional (Fig. 3i). As controls, we tested and found that *N^{spl}/Y; h^{H10}Gal4/+* or *N^{spl}/Y; 109-68Gal4/+* flies displayed facet numbers similar to that in *N^{spl}/Y* (the baseline), indicating that these Gal4 drivers, by themselves, did not modify N^{spl} (Fig. 3i).

To assess whether M8SDΔGro restored R8 patterning and differentiation, we immunostained eye discs for Ato and Sens, respectively. Consistent with the findings of others (Parks et al., 1995), Ato expression is inconsistent in the MF of N^{spl} eye discs, and R8 differentiation is aberrant (note spacing defects in Ato- and Sens-positive cells in Fig. 3a'). In contrast, M8SDΔGro appears to restore Ato expression in the MF, and the spacing of the (Sens-positive) R8 cells (Fig. 3c') appears close to that in the wild type (see Fig. 1).

The retinal defects of N^{spl} are suppressed by reduced E(spl)- or CK2-dosage.

Given that N^{spl} is a gain of function (see above), its retinal defects should be sensitive to *E(spl)*-dosage. Consistent with this prediction, $Df(3R)^{BX22}$ also suppressed the rough and reduced eye of N^{spl} (Fig. 3e), and increased facet numbers (478±19, Fig. 3i) to levels (~470 facets) reported previously (Bose et al., 2006).

We next tested whether the retinal defects of N^{spl} are modulated by reduced CK2 dosage. Based on our finding that knockdown of CK2 antagonizes repression by ectopic M8 (Fig. 2g, h), we reasoned that reduced CK2 levels/activity should attenuate phosphorylation of (endogenous) M8 and thus suppress N^{spl}. Indeed, we find that h^{H10}*Gal4* expression of a *UAS-CK2α-RNAi* construct suppressed the rough and reduced eye of N^{spl} (Fig. 3f), and increased facet numbers to levels (473±23) that approached those with

Df(3R)^{BX22} (Fig. 3i). Similar results were obtained upon co-expression of *UAS-Tik* (Bose et al., 2006), a CK2 dominant-negative construct (data not shown). Similar to our results with M8SDΔGro, expression of the *UAS-CK2α-RNAi* construct restored Ato expression levels at the anterior margin of the MF and patterning of Sens-positive cells posterior to the MF (data not shown).

The suppression of *N*^{spl} by reduced *E(spl)* or CK2 dosage suggest that phosphorylation does augment repression by M8 (Fig. 2). Given the MF-specificity of M8SDΔGro and its effects on Ato and Sens expression, it is likely that its antimorphic activity underlies suppression of *N*^{spl}.

M8SDΔGro suppresses the bristle defects of *N*^{spl}.

We next assessed for modulation of the MC defects of *N*^{spl}. In general, 85-90% of *N*^{spl} males exhibit split and missing MC's, whereas they display ectopic MC's with attenuated (~45%) severity (Fig. 4a, d). Expression of M8SDΔGro potently suppressed the bristle defects of *N*^{spl}, such that only ~1-2% of *N*^{spl} males displayed split, missing or ectopic MC's (Fig. 4d). Moreover, a majority of these flies displayed 4 MC's/scutellum (Fig. 4c), similar to wild type flies. Similar results were obtained upon expression of multiple (14) independent *UAS-m8SDΔGro* insertions (inset in Fig. 4). Consistent with our findings in the eye (see above), a reduction in *E(spl)* dosage (*Df(3R)*^{BX22}), or a reduction of CK2 dosage (CK2α-RNAi) also suppressed the split and missing MC's of *N*^{spl}, but did not modulate the ectopic MC phenotype (Fig. 4d). Under similar conditions, expression of M8*, M8ΔGro or M8SAΔGro did not suppress (or enhance in the case of M8*) the bristle defects of *N*^{spl} (Fig. 4b, d). As controls, we tested and found that *N*^{spl}*/Y; h*^{H10}*Gal4/+*, *N*^{spl}*/Y; UAS-m8SDΔGro/+*, or *N*^{spl}*/Y; UAS-CK2α-RNAi/+* flies all displayed bristle defects whose severity was indistinguishable from that of *N*^{spl} (data not shown). Thus M8SDΔGro is the only E(spl)

variant that suppresses the neural defects of N^{spl}, and its effects are mimicked by reduced CK2- or *E(spl)*-dosage.

Discussion:

The mechanisms by which E(spl) proteins mediate repression have emerged from investigations into loss/gain-of-function mutations, and in vivo assays using site-specific variants. These studies indicate that homo/heterodimers of E(spl) proteins in a complex with Gro antagonize ASC/Ato through DNA-binding and protein-protein interactions. It was initially thought that transcription of *E(spl)* in response to inhibitory N signaling and the accumulation of these proteins was, by itself, sufficient for repression of the neural fate in the non-SOP/R8 cells. Studies on the retinal defects of *E(spl)D* suggest that this is, perhaps, an oversimplification. This mutation encodes a truncated protein (M8*) and increases transcript stability in the MF of the eye disc, the site of expression of endogenous *m8* (Nagel and Preiss, 1999). Despite its inability to bind Gro, M8* severely impairs eye development in the presence of N^{spl}, leading to the proposal that *E(spl)D* is a *gro*-independent hypermorph (Nagel et al., 1999). This possibility is enigmatic since Gro is essential for repression. Moreover, overexpression of wild-type M8 in N^{spl} males does not elicit a reduced eye, suggesting that repression by E(spl) is regulated through the CtD that is lacking in M8*. The severe reduced eye of M8SD, and that CK2-RNAi compromises lateral inhibition implicated this kinase in repression by M8 (Bose et al., 2006; Karandikar et al., 2004). Our studies on the antimorphic activity of the phosphomimetic variant M8SDΔGro, its ability to suppress N^{spl}, and the spatial requirements for the retinal defects of M8SD-vs-M8*, collectively, suggest that spatially controlled, multi-site phosphorylation lies at the heart of repression by M8. These studies provide new insights into repression by M8, and suggest an alternative mechanism for the retinal defects of *E(spl)D*.

Our studies provide strong evidence that M8SDΔGro exhibits key characteristics of an antimorph. It elicits ectopic bristles akin to the loss of *E(spl)C*, the severity of this phenotype is enhanced (albeit modestly) by a deficiency in the *E(spl)C*, and it potently neutralizes the bristle loss phenotype of ectopic M8. Importantly, this antimorphic activity is observed upon expression of all 14 independent M8SDΔGro insertions using two distinct drivers (*scaGal4* and *G455.2*). This, however, is not the case for M8ΔGro or M8SAΔGro, neither of which elicits any overt (bristle) phenotypes and might thus be non-functional. The non-functional (or loss of function) behavior of M8ΔGro has been reported by others as well (Giebel and Campos-Ortega, 1997). While we have tested multiple independent M8ΔGro or M8SAΔGro insertions, it is formally possible that this reflects attenuated expression, or instability in vivo. This caveat does not apply to M8* or M8SDΔGro, since both variants elicit ectopic bristles as previously described for loss of *N* or *E(spl)* functions. Thus the antimorphic activity of M8* in N^{+} flies is mimicked only by M8SDΔGro, among the variants tested. Giebel and Campos-Ortega (1997) presciently suggested that the antimorphic activity of M8* might reflect regulatory influence of the region between Orange and WRPW (lacking in M8*) on E(spl) activity, even though the role of phosphorylation by CK2 was unknown at that time.

It is in N^{spl} that dramatic differences are observed between M8* and M8SDΔGro. Our analysis is of interest because, to our knowledge, M8SDΔGro is the only variant that suppresses the retinal and bristle defects of N^{spl} (Fig. 5a). Several observations suggest that the antimorphic activity of M8SDΔGro underlies suppression of N^{spl}. First, this variant elicits rescue only when expressed anterior to, or within, the MF, and restores Ato- and Sens-expression (Fig. 3). Thus suppression by M8SDΔGro closely correlates, in time and space, to a region of the eye disc wherein the founding R8 photoreceptors are specified, a process that is impaired by N^{spl} (see below). Second, suppression is also seen with reduced

E(spl) dosage (*Df(3R)*^{BX22}, Fig. 3). Of the *E(spl)* members uncovered by this deficiency (*m5*, *m7*, *m8*), only *m8* is expressed in the MF of the eye disc (Cooper et al., 2000; Ligoxygakis et al., 1998). The possibility thus arises that the effects of M8SDΔGro are directed at attenuating this endogenous activity. Third, suppression of *N*^{spl} is also seen upon reduced CK2 levels (Fig. 3), a condition that strongly attenuates repression by ectopic M8 (Fig. 2). This behavior of M8SDΔGro is in contrast to the severe enhancement by M8*. Based on the autoinhibition model (Fig. 1b), one would have predicted that M8SDΔGro should have enhanced, rather than suppressed, *N*^{spl}. A refinement of the autoinhibition model (see below) and a fundamental difference in N signaling between the bristle and the eye may provide an explanation for these unexpected results.

In the case of the MC's, expression of ASC is under the control of pre-pattern factors, but not N signaling (reviewed in Gomez-Skarmeta et al., 2003). Consequently, loss of N signaling compromises lateral inhibition, and elicits ectopic bristles, as is seen with the loss of *E(spl)C*. While all E(spl) members repress as dimers (Alifragis et al., 1997), the underlying reason(s) have remained unclear. It has been suggested that dimers of bHLH proteins, such as Drosophila Hairy or human Hes6, might be necessary for proper Gro-recruitment, possibly by the presentation of dual WRPW-motifs (Belanger-Jasmin et al., 2007; Jennings et al., 2008). If so, dimerization of M8* or M8SDΔGro with endogenous E(spl) proteins would impair Gro-recruitment and repression. This possibility is supported by our findings that M8SDΔGro potently neutralizes repression by ectopic M8 (Fig. 2), and that these proteins form direct complexes (in yeast two-hybrid assays, data not shown). Thus the antimorphic behavior of M8* and M8SDΔGro in *N*$^{+}$ might reflect neutralization of endogenous E(spl).

The divergent behaviors of M8SDΔGro and M8* in *N*^{spl} can be explained given the biphasic nature of N signaling during R8 specification, the first step in retinal histogenesis

(reviewed in Frankfort and Mardon, 2002; Hsiung and Moses, 2002). Anterior to the MF, N elicits *ato* expression ('proneural enhancement'), whereas in the MF it elicits *E(spl)* expression and lateral inhibition (Ligoxygakis et al., 1998). These functions are differentially affected in N^{spl}. It was initially thought that N^{spl}, which does not alter *E(spl)* expression, is compromised for *ato* expression (Nagel and Preiss, 1999). However, analysis of clones of N^{spl} (in N^{spl}/+ females) indicates that N^{spl} renders R8 precursors sensitive to inhibitory N activity (Li *et al.*, 2003). This N activity negatively impacts R8 specification and differentiation. As a result, inductive signals such as Hedgehog and Decapentaplegic, which are secreted by differentiating photoreceptors posterior to the MF, are defective in N^{spl} (Li et al., 2003). These signals are necessary for *ato* expression anterior to the MF. Thus, the reduced *ato* expression in N^{spl} reflects non-autonomous effects.

The identification of enhancers/suppressors of N^{spl} also supports the notion that this allele renders R8's sensitive to inhibitory N signaling. For example, the retinal defects of N^{spl} are suppressed by halved dosage of *E(spl)C* or *Dl* (Parks et al., 1995), suggesting that under these conditions lateral inhibition is attenuated in the sensitized R8's. Similar effects are seen with halved dosage of *sca* (Baker et al., 1990; Brand and Campos-Ortega, 1990), reflecting its role in repression of *ato* during formation of intermediate groups (Baker et al., 1996). In contrast, reduced dosage of *da*, a co-activator of Ato/ASC, enhances N^{spl} (Brand and Campos-Ortega, 1990). N^{spl} is likely to also render the SOP's sensitive to inhibitory N signaling, given its diverse bristle defects (Brennan et al., 1997). Thus, N^{spl} provides a background that is highly sensitive to levels/activities of the Ato/ASC activators as well as the E(spl) repressors.

It is in this (N^{spl}) background of reduced Ato that M8* displays hypermorphic effects. It is thought that the Ato-positive feedback loop, which mediates proneural gene 'self stimulation' in the future R8 cells (Sun et al., 1998), involves cooperative interactions

between adjacently positioned Ato+Da dimers (Powell et al., 2004). Given the preferentially strong interaction of M8* with Ato and Da (Nagel et al., 1999; Nagel and Preiss, 1999), it seems reasonable to suggest that M8* might impair the Ato feedback loop. Consistent with this possibility, Ato levels precipitously decrease anterior to the MF in N^{spl}*/Y; E(spl)D/+* or in N^{spl}*/Y;* h^{H10}*Gal4/UAS-m8** discs (Karandikar et al., 2004; Nagel and Preiss, 1999). Given the already low Ato levels in N^{spl} (Fig. 3), this impairment will further attenuate Ato activity, which would then be insufficient to support R8 specification and elicit loss of the eye field. Thus the behavior of M8* in N^{spl} is unlikely to reflect a hyperactive repressor (that functions without Gro), but instead reflects its ability to prevent upregulation of *ato* expression in the sensitized R8 precursors. Consistent with this, the effects of *E(spl)D* in N^{spl} females are enhanced by mutations in *ato* or *da* (Nagel and Preiss, 1999).

This interpretation accounts for the enhancement of N^{spl} by M8*, but not for its suppression by M8SDΔGro. Based on the autoinhibition model (Fig. 1), these two variants were expected to elicit similar outcomes. To resolve this paradox, we assessed the spatial requirements for the reduced eye of M8* in N^{spl} versus M8SD in N^{+} (Fig. 5b). Surprisingly, expression of M8* in the MF of N^{spl} males (with *109-68Gal4*) has minimal effects, i.e., these flies display N^{spl} eyes (Fig. 5c). Thus, once the 'Ato-self stimulatory' loop is established and Ato levels rise M8* loses potency. This finding is in line with our suggestion that the dominant effects of M8* reflect impaired 'Ato-self-stimulation'. The reduced eye of M8SD in N^{+} is also specific to its expression domain (Fig. 5b). Unlike the strong loss of the eye field when expressed in the MF (*109-68Gal4,* Fig. 1) or *scaGal4* (Karandikar et al., 2004), M8SD is ineffective when expressed anterior to the MF (h^{H10}*Gal4*, Fig. 5b, c). We interpret this MF specificity in the context of secondary phosphorylation.

The CK2 site in M8 resides in a region that harbors additional Ser residues (Fig. 5d). Importantly, these residues are conserved in Drosophila M8/5/7 and in human/murine Hes6.

In the case of Hes6, secondary phosphorylation appears necessary for function in vivo (Belanger-Jasmin et al., 2007). If this were to apply to Drosophila M8 as well, multi-site phosphorylation would be a prerequisite for repression. If CK2 phosphorylation served as a 'priming' step, ectopic M8SD would be precociously subject to multi-site phosphorylation, precipitating its strong repressor activity. This effect would be restricted, in that only when expressed in the MF (stage 2/3, Fig. 5b) would it demonstrate a phenotype. Its inability to elicit a reduced eye anterior to the MF (*h*^{H10}*Gal4*, Fig. 5c) might reflect a spatial context where the additional participating kinases have not yet been activated. Absent the secondary phosphorylation, M8SDΔGro might remain autoinhibited, i.e., refractory for Ato binding. When assayed in yeast (two-hybrid), M8SD interacts more strongly with Ato, as compared to M8 (Karandikar et al., 2004), and the possibility remains that this variant is predisposed to secondary phosphorylation by endogenous yeast kinases, or that the observed interaction does not reflect the in vivo situation. In any case, M8SDΔGro retains the capacity to dimerize with, and neutralize, endogenous E(spl), and this antimorphic activity would enable Ato levels to rise to a threshold sufficient for specifying the R8 fate. This interpretation is further supported by our findings that Ato levels in the MF of *N*^{spl} discs are significantly enhanced by M8SDΔGro (Fig. 3c'), and that decreased *E(spl)*- or CK2-dosage also suppress the retinal/bristle defects of *N*^{spl} (Figs. 3, 4).

Examination of M8 indicates that Ser^{151} meets the consensus (Px**S**P, Fig. 5d) for the mitogen-activated protein kinases (MAPK). Indeed, diphospho-ERK (activated MAPK) is detected in intermediate group cells in the MF, leading to the suggestion that activity of the EGF receptor is required for lateral inhibition of R8 cells to occur promptly (Lesokhin et al., 1999). It is, however, unknown if this regulation reflects modification of endogenous M8. The remaining sites in M8 might be modified by CK1 (Ser^{154}) and GSK3 (Ser^{155}), whose site recognition is influenced by the phosphorylation status of adjacent residues (reviewed in

Roach, 1991). So far, R8 defects have not been described for mutations in either gene. It is noteworthy that the activities of CK2, CK1, GSK3 and the phosphatase PP2A regulate the clock protein Period (Kim and Edery, 2006; Lin et al., 2002; Sathyanarayanan et al., 2004). In this regard, PP2A has been implicated in N signaling (Abdelilah-Seyfried et al., 2000). Irrespective if it is hierarchical or independent, multi-site phosphorylation of M8 might represent a developmental 'checkpoint' enabling repression to only occur in a precise spatial context and without a temporal lag.

The most parsimonious interpretation of our studies is that it is the bypass of the multi-site phosphorylation, rather than Gro-binding, that underlies the dominant behavior of M8*. Our studies provide a putative mechanism to address the paradoxical behavior of M8*, and further illustrate the complex regulatory role of phosphorylation of M8. Future efforts to parse which of these Ser residues are modified, in addition to Ser^{159} (the CK2 site), the identity of the kinase(s), and the signaling processes will be required to more fully define the mechanism by which spatially controlled multi-site phosphorylation regulates repression by M8 during lateral inhibition and neural selection.

Methods:

Plasmid construction and germline transformations.

The construction of variants of M8 harboring Ala/Asp in place of Ser^{159} (M8SA and M8SD) has been described previously (Trott et al., 2001). The Gro-compromised variants were generated by the substitution of a stop codon in place of the W^{176} of the WRPW motif. All constructs were sequenced to confirm the presence of only the intended mutations. For *in vivo* expression, cDNA's were cloned into the EcoRI and BamHI sites of the plasmid pUAST (Brand and Perrimon, 1993), and germ line transformants were generated as described

(Rubin, 1983). w^+ progeny were identified and the location of insertions was determined via crosses to lines harboring chromosomes carrying dominant visible markers. At least 6-14 independent insertions of each construct have been used in these studies. *UAS-m8**, *UAS-CK2α-RNAi* and *UAS-Tik* lines have been previously described (Bose et al., 2006; Nagel et al., 1999).

Protein-protein interactions.

Protein-protein interactions were analyzed by the LexA-based version of the yeast interaction trap (Gyuris et al., 1993). Bait and prey constructs were expressed as C-terminal fusions with the DNA-binding domain of LexA or the activation domain (AD) of B42, and interactions were assessed in yeast EGY048. *LacZ* activity was determined for at least three independent transformants, each in triplicate, employing XGal or ONPG as substrates, as described (Trott et al., 2001). *LacZ* activity was determined using the formula $1000xOD_{420}/(TxVxOD_{600})$, where T is minutes and V is the concentration factor of the assay.

Fly stocks, crosses and phenotypes.

Flies were raised at 24^{o}C on standard Yeast-Glucose medium. The *Gal4* drivers used in these studies were generously provided by other researchers or obtained from the Bloomington Stock Center (denoted by the prefix B). These drivers are *G455.2* (Giebel and Campos-Ortega, 1997), *scaGal4* (Nakao and Campos-Ortega, 1996), *109-68Gal4* (Jarman and Ahmed, 1998), and h^{H10}*Gal4* (Huang and Fischer-Vize, 1996). *UAS-m8**, and *UAS-ato* flies were gifts of Drs. Anette Preiss and Yuh Nun Jan, respectively.

All crosses were performed at 24^{o}C. Fly heads were dehydrated by sequential passes through a graded alcohol series (25-50-75-absolute) and finally through Hexamethyldisalizane. Heads were mounted on EM stubs, dried for 24 hours, sputter coated with gold, and examined with a JEOL-6400 scanning electron microscope at an

accelerating voltage of 20 kV. For bristle phenotypes, newly eclosed adults were photographed. For quantitative analysis of the bristle phenotypes, multiple crosses were established (≥triplicates), and adults were scored for bristle artifacts. In every case multiple independent insertions of *UAS*-constructs were used.

Immunostaining and confocal microscopy.

Imaginal discs were isolated from late third instar larvae and processed as described (Kavler et al., 1999) with modifications. Discs were fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 15 minutes, and washed three times with PBS containing 0.1% Triton X-100 (PBS-TX). The discs were incubated for 12 hours at 4^{o}C in PBS-TX containing 5% normal goat serum and then immunostained. The following antibodies were used in this study: rabbit anti-Ato (1:1000, gift of Yuh Nun Jan), guinea pig anti-Sens (1:800, gift of Hugo Bellen) and mAb-ELAV (1:1000, DHSB, Iowa City). Secondary antibodies (Molecular Probes) were goat-anti rabbit-IgG coupled to Alexa Fluor 594 (1:1000), donkey anti-guinea pig-IgG coupled to Alexa Flour 488 (1:1000), and goat anti-mouse-IgG coupled to Alexa Fluor 594 (1:1000). Discs were mounted in Vectashield. An Olympus FluoView (FV1000) was used for confocal imaging. Images in Figs. 1 and 3 represent scans acquired every 1 μm along the apicobasal axis of the discs and then compressed as a Z-stack.

Acknowledgments:

We thank Philip Keeting for comments on the manuscript, and Sophia Zhang for technical assistance. We are indebted to Anette Preiss for *UAS-m8** flies, Yuh Nung Jan for *UAS-ato* and *109-68Gal4* flies and anti-Ato antibodies, Hugo Bellen for anti-Sens antibodies, J. Fischer-Vize for $h^{H10}Gal4$ flies and Christos Delidakis for the $Df(3R)^{boss14}$ and $Df(3R)^{BX22}$ flies. We thank Diane Schwegler-Berry (NIOSH, CDC) for assistance with electron microscopy.

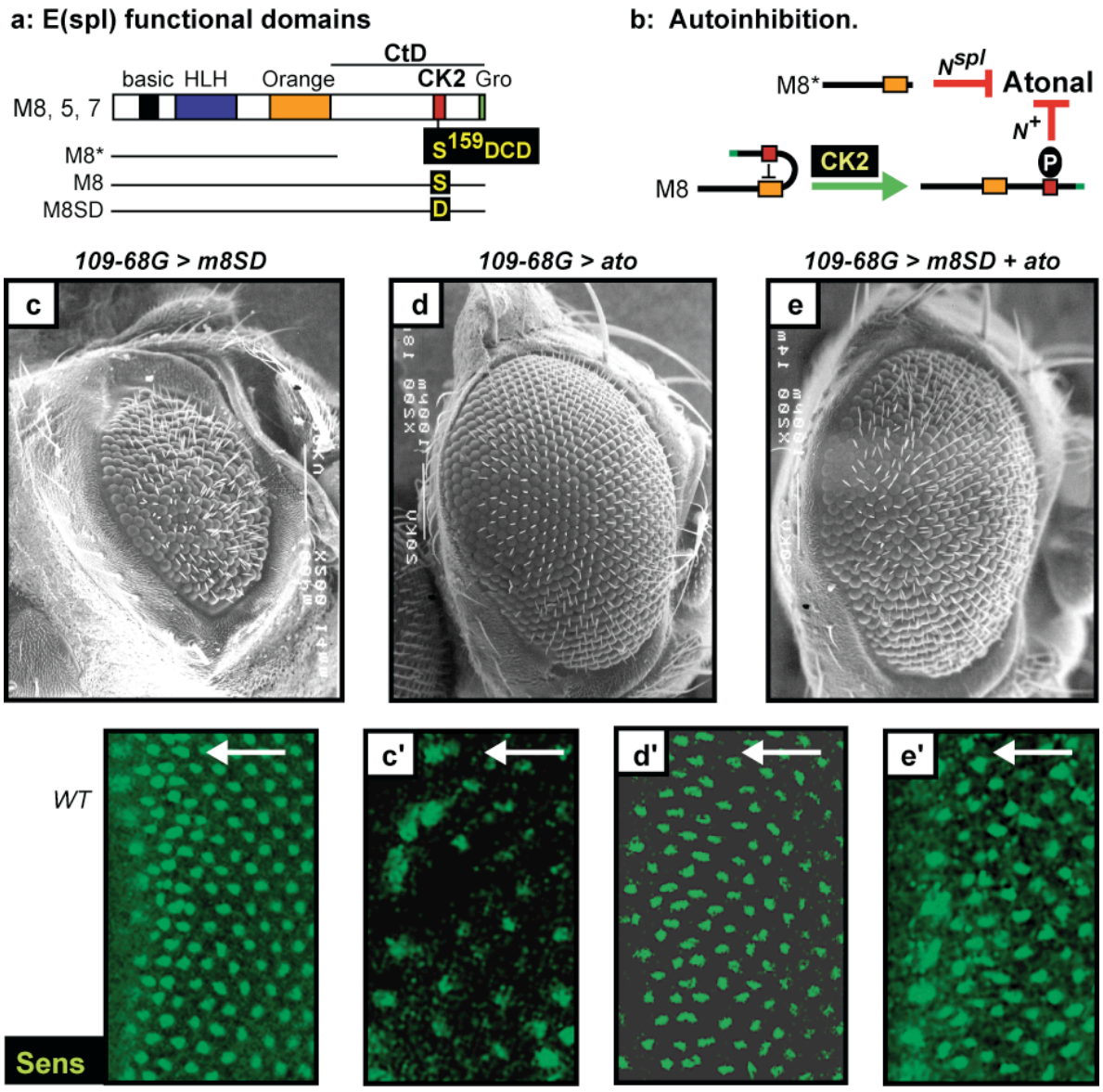

Figure 1. Role of phosphorylation of M8 and suppression of eye defects of M8SD with increased dosage of Ato. (a) Conserved domains in M8, M5, and M7. The C-terminal domain (CtD) harbors a CK2 phosphorylation site (SDCD). (b) Regulation of the antagonism of Ato upon phosphorylation of M8 (color of boxes correspond to those in panel a). The ability of M8SD to antagonize Ato and block eye development occurs in N^+ flies,

whereas M8* requires N^{spl}. (c-e) The eye defects of M8SD are suppressed by increased Ato dosage. The genotypes are, (c) *109-68Gal4/+; UAS-m8SD/+*, (d) *109-68Gal4/+; UAS-ato/+*, and (e) *109-68Gal4/+; UAS-m8SD/UAS-ato*. Magnifications are 200x. (c', d', e') Eye discs were immunostained with α-Sens; WT denotes wild type. Arrows denote the direction of MF progression. The genotype of discs in c', d' and e' are as in panels c, d and e, respectively.

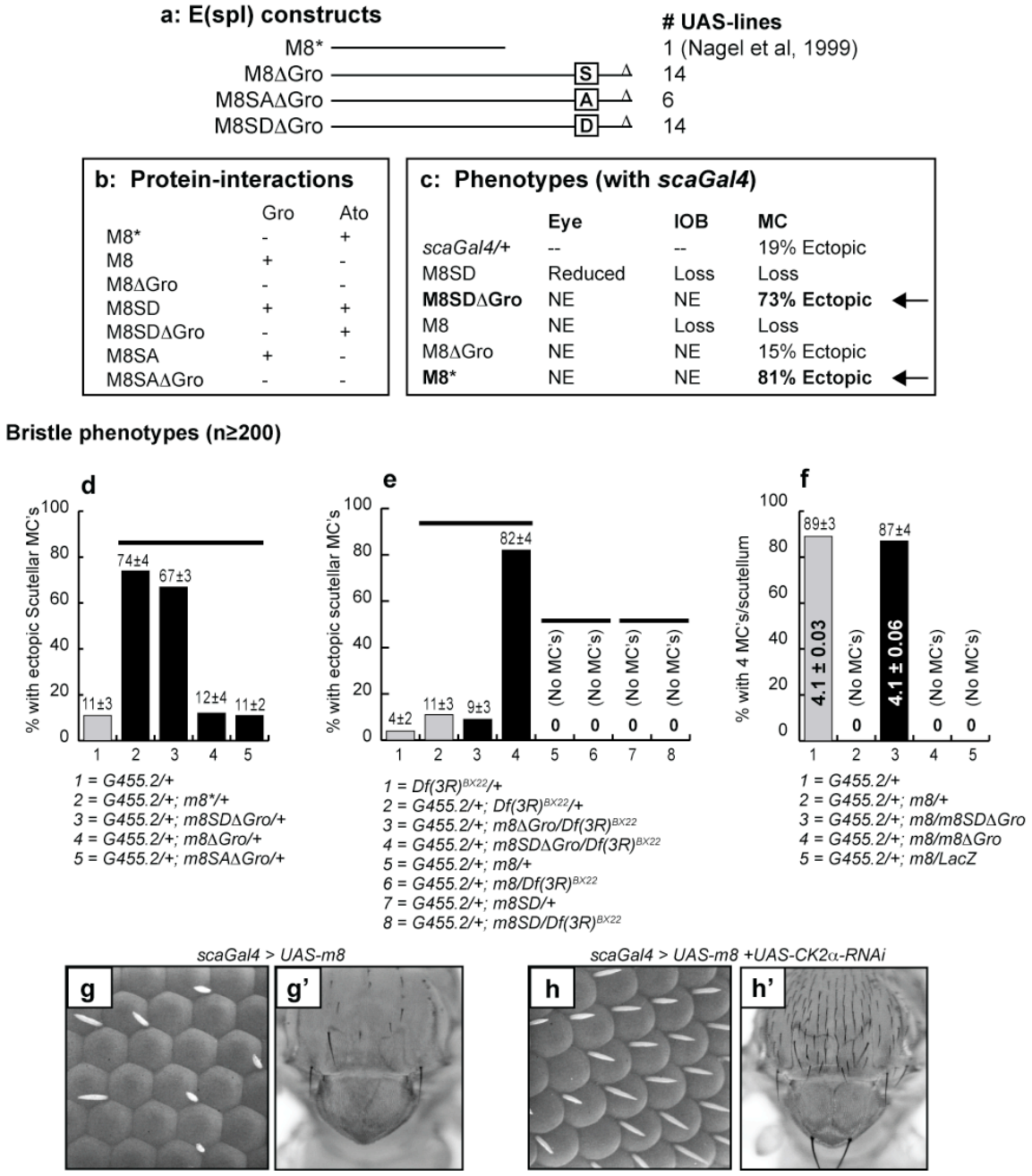

Figure 2. M8SDΔGro exhibits antimorphic activity. (a) Schematic of variants. The triangles denote removal of WRPW (ΔGro), and S/A/D represent variants harboring Ser/Ala/Asp at position 159, respectively. (b) Yeast two hybrid interactions. (c) Crosses

between *scaGal4* and *UAS-constructs* were conducted at 24^{o}C; NE denotes no effect. The inability of M8* to elicit any eye defects is because crosses were conducted in N^{+} flies. (d-f) Bristle phenotypes; the genotypes are as indicated. (g-h) The IOB, MC and mc loss of ectopic M8 are antagonized by co-expression of a *UAS-CK2α-RNAi* construct.

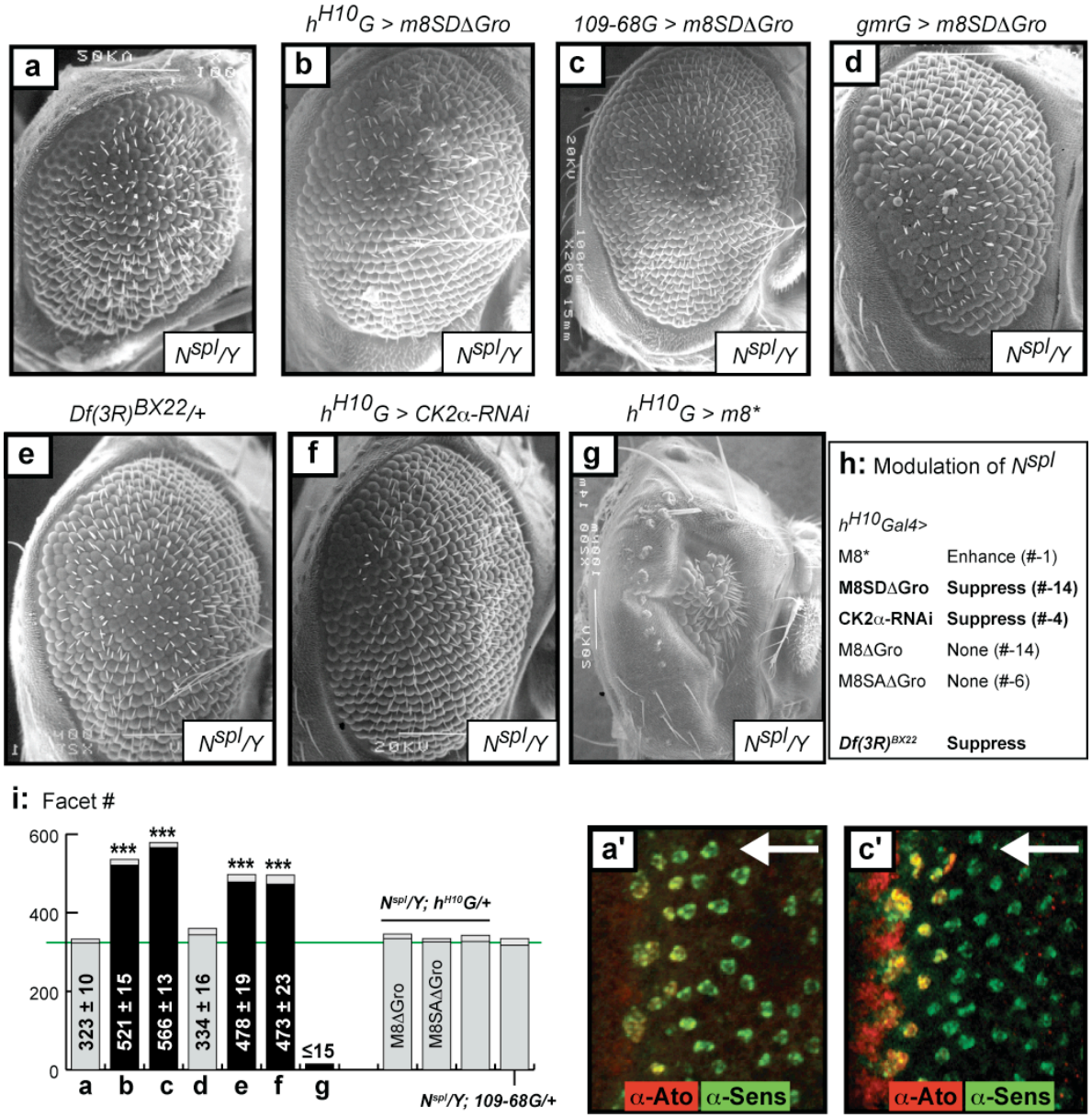

Figure 3. M8SDΔGro suppresses the retinal defects of N^{spl}. (a-g) Eye phenotypes; the Gal4 drivers are, $h^{H10}G$ ($h^{H10}Gal4$), *109-68G* (*109-68Gal4*), and *gmrG* (*gmrGal4*). The genotypes are, (a) N^{spl}/Y, (b) N^{spl}/Y; *UAS-m8SDΔGro/+*; $h^{H10}Gal4/+$, (c) N^{spl}/Y; *109-68Gal4/UAS- m8SDΔGro*, (d) N^{spl}/Y; *UAS- m8SDΔGro/+; gmrGal4/+*, (e) N^{spl}/Y; $Df(3R)^{BX22}/+$, (f) N^{spl}/Y; *UAS-CK2α-RNAi/+*; $h^{H10}Gal4/+$, and (g) N^{spl}/Y; *UAS-m8*/+*; $h^{H10}Gal4/+$.

Magnifications are 200x. (h) Summary of effects on N^{spl}. The number in parenthesis denotes number of insertions tested. (i) Quantitative analysis of suppression of N^{spl} (facet counts). The numbers correspond to the genotypes in panels a-g, and asterixes denote *P*-values <0.0001. (a', c') Eye discs were immunostained with α-Ato and α-Sens. Genotypes of discs in a' and c' correspond to those in panels a and c, respectively, and arrows denote MF progression.

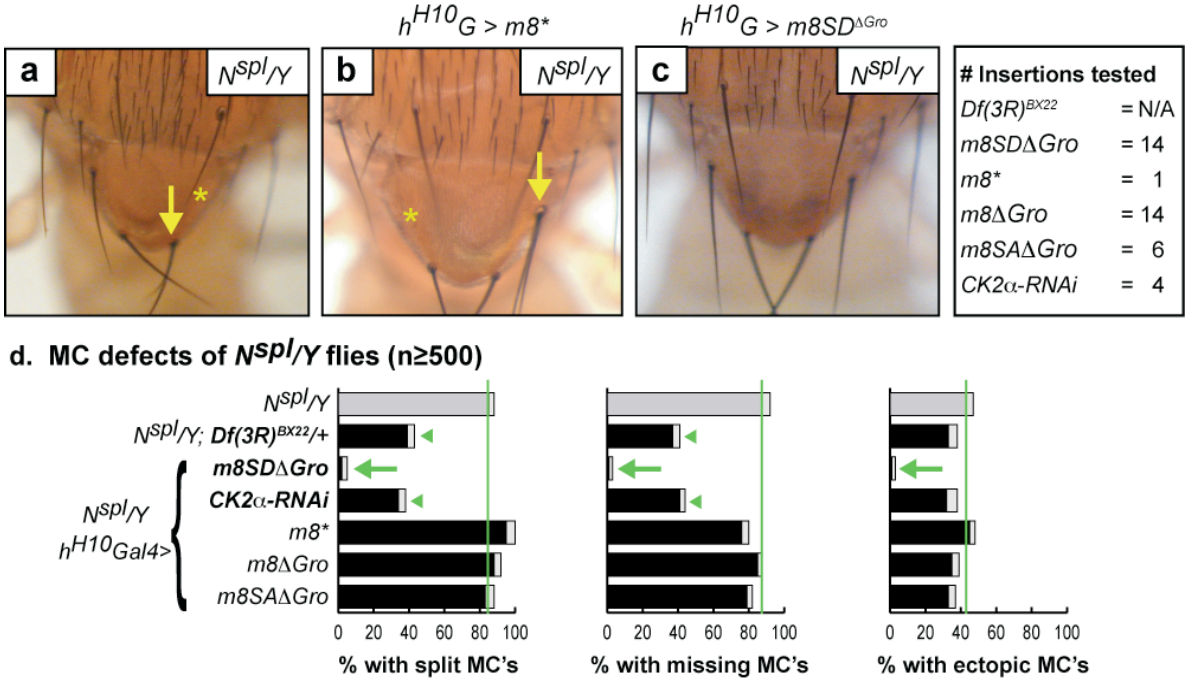

Figure 4. M8SDΔGro suppresses the bristle defects of N^{spl}. (a-c) Nota of flies. Genotypes are, (a) N^{spl}/Y, (b) N^{spl}/Y; *UAS-m8*/+*; h^{H10}*Gal4*/+, and (c) N^{spl}/Y; *UAS-m8SDΔGro/+*; h^{H10}*Gal4*/+. Asterisks denote missing bristles, and arrows denote split bristles, both characteristic of N^{spl} males. (d) Modulation of N^{spl} bristle defects by M8-variants, reduced *E(spl)*- or CK2-dosage. *UAS* constructs were expressed with h^{H10}*Gal4*. Inset shows the number of insertions tested; in all cases ≥500 flies of the relevant genotype were assessed for MC defects. Arrows denote strong suppression and arrowheads denote moderate suppression.

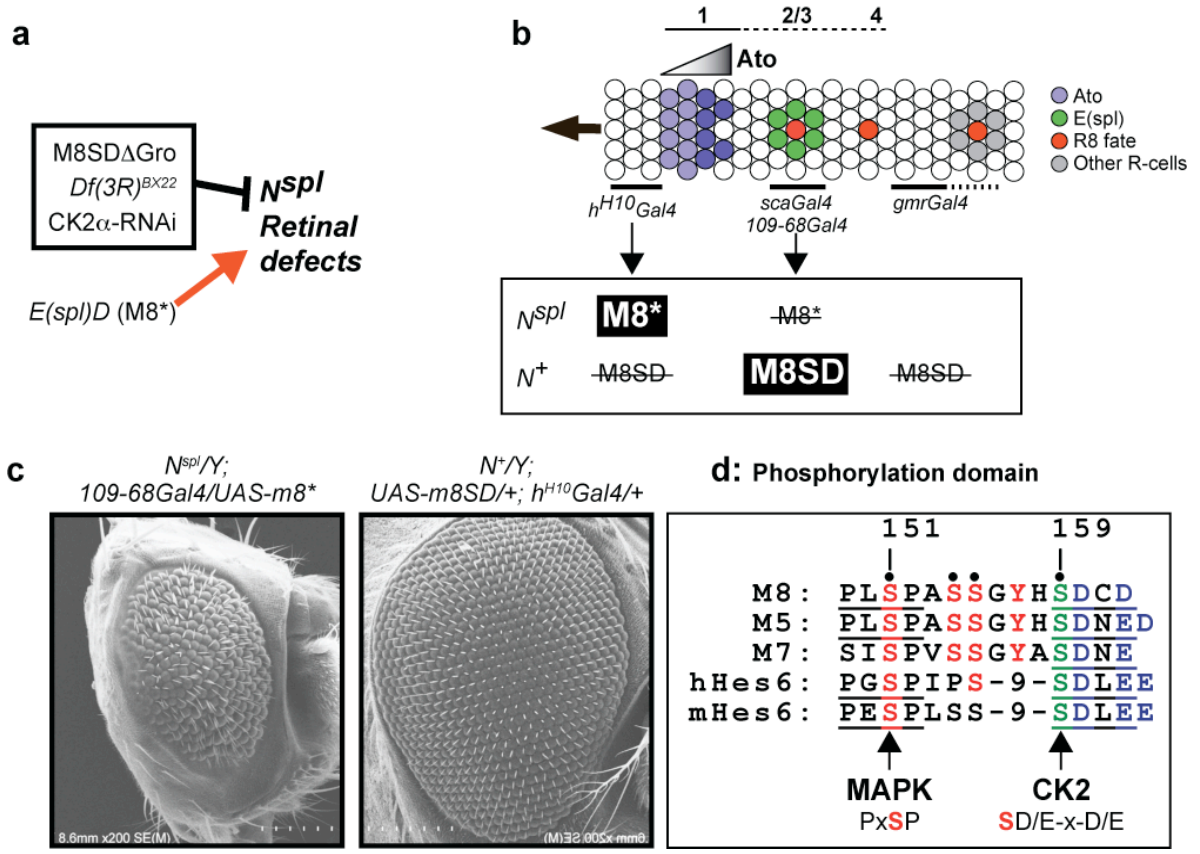

Figure 5. Modulation of N^{spl} and MF-specificity. (a) M8SDΔGro, reduced E(spl)- or reduced CK2-dosage suppress the retinal defects of N^{spl}, whereas M8* enhances. (b) MF specificity of M8* and M8SD. Schematic of cells spanning stages 1-4 of the MF are shown relative to the Gal4 drivers. *ato* expression in response to N signaling ('proneural enhancement') is denoted as a shaded triangle. Retinal phenotypes of M8* (in N^{spl}) and M8SD (in N^{+}). Black box denotes a region of expression leading to a reduced eye and strikethrough denotes no retinal phenotypes. (c) Adult eye phenotypes of the indicated genotypes. (d) Conserved secondary phosphorylation sites (black dots) in Drosophila E(spl) and human (h) and murine (m) Hes6. Numbers above alignment refer to the M8 protein. Ser^{159} and Ser^{151} meet the consensus recognition motifs for CK2 and MAPK, respectively.

Chapter 3

Evidence that the C-terminal domain (CtD) autoinhibits neural repression by Drosophila E(spl)M8.

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Abstract:

Analysis of the retinal defects of a CK2 phosphomimetic variant of E(spl)M8 (M8S^{159}D) and the truncated protein M8* encoded by the *E(spl)D* allele, suggest that the non-phosphorylated CtD 'autoinhibits' repression. We have investigated this model by testing for inhibition (in '*trans*') by the CtD fragment in its non-phosphorylated (M8-CtD) and phosphomimetic (M8SD-CtD) states. In N^{+} flies, ectopic M8-CtD compromises lateral inhibition, i.e., elicits supernumerary bristles as with loss of N signaling. This antimorphic activity of M8-CtD strongly rescues the reduced eye and/or bristle loss phenotypes that are elicited by ectopic M8SD or wild type M8. Additionally, the severely reduced eye of N^{spl}*/Y; E(spl)D/+* flies is also rescued by M8-CtD. Rescue is specific to the time and place, the morphogenetic furrow, where 'founding' R8 photoreceptors are specified. In contrast, the phosphomimetic M8SD-CtD, which is predicted to be deficient for autoinhibition, exhibits significantly attenuated or negligible activity. These studies provide evidence that autoinhibition by the CtD regulates M8 activity in a phosphorylation-dependent manner.

Introduction:

N signaling has been intensively studied during Drosophila neurogenesis, a process leading to stereotyped patterning of the compound eye and bristles (Baonza and Freeman, 2001; Bray, 2006; Campos-Ortega, 1997). Neural development initiates with the expression of *atonal (ato*) or the *achaete-scute* complex (*ASC*), which encode basic-helix-loop-helix (bHLH) transcription factors, the proneural activators (Cubas et al., 1991; Heitzler et al., 1996; Jarman et al., 1995; Jarman et al., 1994; Modolell and Campuzano, 1998; Skeath and Carroll, 1991). *ato* is required for eye development and *ASC* for the bristle. During early neurogenesis, these activators are expressed in groups of cells called the proneural clusters (PNC's; Calleja et al., 2002; Dambly-Chaudiere and Vervoort, 1998; Frankfort and Mardon, 2002; Gibert and Simpson, 2003; Hsiung and Moses, 2002). However, only a fixed number of cells from each PNC are selected to adopt the neural fate, while the others are redirected to an alternative fate. This selection begins when one cell of a PNC gains an advantage by expressing the highest level of Ato/ASC. This cell is destined to form the 'founding' R8 photoreceptor or the bristle sensory organ precursor (SOP), and inhibits other PNC cells from adopting the (default) R8/SOP fate. This N-dependent process is termed lateral inhibition (Lehmann et al., 1983; Simpson, 1990). The future R8/SOP expresses Delta (*Dl)* at a higher level, and activates N in adjoining cells of the PNC. As a result, N is cleaved and its intracellular domain (N^{ICD}) then elicits transcription of the *Enhancer of split Complex* (*E(spl)C* (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Schrons et al., 1992). In cells receiving this inhibitory N signal, E(spl) repressors complex with the co-repressor Groucho (Gro) and antagonize Ato/ASC, thereby preventing them from adopting the R8/SOP fate.

Given the importance of E(spl) for lateral inhibition, numerous studies have sought to define their modes of action. These studies have turned out to be complicated, in part,

because the *E(spl)C* encodes seven bHLH proteins with similar functional domains, and mutations affecting each transcription unit have been unavailable. Earlier studies that overexpression of E(spl) proteins elicits generalized bristle loss (Giebel and Campos-Ortega, 1997; Nakao and Campos-Ortega, 1996) raised the possibility of functional redundancy (Cooper et al., 2000). A more recent model, termed 'the protein tether' (Giagtzoglou et al., 2003), proposes that, in addition to DNA-binding, E(spl) proteins mediate repression by directly interacting with enhancer-bound Ato/ASC. One prediction of this model is that repression should reflect E(spl) dosage. Studies in the eye have produced mixed results. Three E(spl) members (M8, Mγ and Mδ) are expressed in the morphogenetic furrow (MF), and mediate R8 selection (Ligoxygakis et al., 1999). However, overexpression of only Mδ antagonizes Ato and elicits loss of the R8's, no such effects were seen with M8 or Mγ (Ligoxygakis et al., 1999; Ligoxygakis et al., 1998; Nagel et al., 1999). This does not reflect sub-threshold expression levels, because ectopic M8 elicits potent bristle loss (Giebel and Campos-Ortega, 1997). A predominant role for M8 in R8 selection and eye development is highlighted by *E(spl)D*, a unique dominant allele of *m8* that abrogates eye development in the presence of N^{spl}. It was, in fact, this genetic interaction that led to the identification of the *E(spl)C* (Welshons, 1956).

The *E(spl)D* allele harbors two lesions; one stabilizes the *m8* transcript, and the second results in a truncated protein called M8* (Nagel et al., 1999; Tietze et al., 1992). M8* retains the basic, HLH and Orange (also an HLH) domains (see Fig. 1), but lacks 56 C-terminal residues (CtD) including the penultimate WRPW tetra-peptide, which abolishes Gro-binding (Paroush et al., 1994). Despite this critical defect, *E(spl)D* dominantly elicits loss of R8 cells by exacerbated interaction with, and antagonism of, Ato (Nagel et al., 1999). Importantly, the R8/eye defects of *E(spl)D* are mimicked by expression of a *UAS-m8** construct, but not by full length M8, strongly suggesting that the truncation triggers dominant

behavior. However, removal of just the WRPW motif does not elicit dominant activity on otherwise full length M8 (Giebel and Campos-Ortega, 1997; Nagel et al., 1999). These paradoxical findings, led Giebel and Campos-Ortega (Giebel and Campos-Ortega, 1997) to presciently suggest that 'the region between Orange and WRPW may have regulatory influence on repressor activity'. Our previous studies indicate that this regulatory influence involves phosphorylation of M8.

We had uncovered that protein kinase CK2 phosphorylates M8 at Ser^{159} (Trott et al., 2001), in its CtD, a region missing in M8*. Expression of a CK2 phosphomimetic variant, $M8S^{159}D$ (M8SD), led to a severely reduced eye due to exacerbated antagonism of Ato (Karandikar et al., 2004). These effects closely mimicked the eye defects of M8* previously described by others (Nagel et al., 1999). In addition, the strength of binding of M8SD with Ato was virtually identical to that of M8* and Ato. This result suggested that the CtD in its phosphorylated state (M8SD) does not contribute to Ato-binding, consistent with the role of Orange in Ato-binding (Nagel et al., 1999). In contrast, wild type M8 did not bind Ato. These findings and the suggestion of Giebel and Campos-Ortega (see above), led us to propose that non-phosphorylated M8 was 'autoinhibited' by its CtD. In this case, an intra-molecular (*'cis'*) interaction of the non-phosphorylated CtD with HLH/Orange prevented binding of wild type M8 to Ato (Fig. 1). Phosphorylation by CK2 would displace the CtD to expose Orange, and permit binding to Ato, an interaction that is required for repression of the R8 fate. The absence of the CtD in M8* would bypass autoinhibition and trigger dominant activity against Ato and the R8 fate. However, direct evidence for autoinhibition by the non-phosphorylated CtD was lacking.

We describe here studies employing Gal4-*UAS* driven overexpression of the CtD peptides, and their effects on neural patterning. Since phosphorylation of M8 is required for R8 and SOP selection (Bose et al., 2006), we hypothesized that ectopic CtD might bind (in

'trans') to the non-inhibited state of M8 and impair repression. To distinguish the effects of phosphorylation, we used two CtD-variants; M8-CtD retains the phosphorylation site and should possess autoinhibitory activity, whereas M8SD-CtD should lack such activity by virtue of the phosphomimetic Asp at the CK2 site. Our studies on rescue of the eye and bristle defects of ectopic M8 and M8SD, and the reduced eye of N^{spl}*/Y; E(spl)D/+* are consistent with autoinhibition. The implications of these findings are discussed.

Results:

The CtD fragment is phosphorylated by CK2 and binds Gro in vitro.

As stated above (see Introduction), studies on M8SD and M8* suggested that phosphorylation of the CtD would overcome its ability to 'autoinhibit', perhaps, by preventing interaction with the HLH or Orange domains (see Fig. 1a). If so, over-expressed CtD might bind to phosphorylated (non-inhibited) M8 and impair repression, even though such a '*trans*' interaction is expected to display first-order, rather than zero-order kinetics (as with full-length M8). We generated two variants of the CtD (Fig. 1b). M8-CtD is phosphorylatable and should retain autoinhibitory activity, whereas the phosphomimetic variant M8SD-CtD should exhibit lower potency or lack such activity.

We first characterized the in vitro interactions of these 56 residue peptides with CK2 and Gro. Phosphorylation of CtD-peptides (as GST-fusions) was tested using CK2 (the $\alpha 2\beta 2$ holoenzyme) purified from Drosophila embryos (Kahali et al., 2008). In assays employing limiting amounts of CK2, we find that M8-CtD is readily phosphorylated, whereas M8SD-CtD or GST-alone are not (Fig. 1c, and data not shown). Phosphorylation was not observed in (mock) reactions lacking the enzyme (data not shown), and is specific because

this purified enzyme is potently inhibited by Heparin, a CK2-specific inhibitor (Kahali et al., 2008). It appears that the specificity of CK2 for Ser^{159} is not altered in the CtD peptides. We also assessed and found that both CtD's interact equivalently with Gro in yeast (Fig. 1d), consistent with the role of WRPW in mediating this interaction (Fisher et al., 1996). Therefore, the two motifs resident in the CtD, CK2-phosphorylation and Gro-binding (Fig. 1b), appear to retain function in the absence of the basic, HLH and Orange domains.

M8-CtD elicits stronger ectopic bristle defects than does M8SD-CtD.

We next assessed if over-expression of M8-CtD, but not M8SD-CtD, elicits ectopic macrochaetes (MC's), a phenotype characteristic of impaired lateral inhibition (Brennan et al., 1997), and one seen with antimorphic or dominant-negative (DN) variants of M8 (Giebel and Campos-Ortega, 1997). For in vivo analysis, the *UAS-m8-CtD* and *UAS-m8SD-CtD* constructs were expressed using the Gal4-*UAS* system (Brand and Perrimon, 1993). To eliminate position effects, multiple independent lines harboring *UAS*-constructs were generated (Fig. 2a), and expression was driven with the enhancer trap *G455.2* that elicits Gal4 expression in the PNC's that give rise to the four scutellar MC's (Giebel and Campos-Ortega, 1997; Hinz et al., 1994).

In the case of *G455.2/+* flies, by themselves, ~11% display ectopic scutellar MC's; this number was used as the baseline for comparing the effects of ectopic M8-CtD or M8SD-CtD. We find that expression of M8-CtD elicited ectopic MC's on the scutellum of 62-78% of the flies, with an average penetrance of ~70% (Fig. 2b). Expression of M8SD-CtD also led to ectopic scutellar MC's, but with a generally lower penetrance; it ranged from 31-54%, with an average of ~41% (Fig. 2b). On their own, the intrinsic MC defects in *UAS-m8-CtD/+* or *UAS-m8SD-CtD/+* lines were ≤5% (Fig. 2b), indicating that expression was required. Analyses of 5 independent lines revealed that the penetrance of the ectopic MC phenotype of M8-CtD (lines S1-S5) was generally higher than those of M8SD-CtD (lines D1-D5, Fig.

2b). These studies raised the possibility that M8SD-CtD is less efficient at autoinhibition, perhaps reflecting kinetic effects. A direct assessment of expression levels of the CtD peptides was precluded by the absence of suitable antibodies. We therefore used additional assays to compare the activities of the CtD peptides in vivo (see below).

M8-CtD more strongly suppresses the eye and bristle defects of ectopic M8SD.

We have previously reported that expression of M8SD with *109-68Gal4* elicits a reduced eye and loss of the interommatidial bristles (IOB's, Karandikar et al., 2004). This Gal4 driver elicits expression within the MF, where endogenous E(spl) mediates selection of R8 cells. The reduced eye of M8SD also displays defects in ommatidial (facet) size and patterning (Fig. 3a, a'). These defects are strongly rescued by co-expression of *UAS-ato*, an in vivo target of M8SD (Kahali et al., 2009). Importantly, rescue does not involve competition between two *UAS*-constructs for a limiting amount of Gal4, because co-expression of *UAS-LacZ* does not modulate the eye defects of *109-68Gal4>UAS-m8SD* flies. We therefore tested for rescue of the M8SD reduced eye by co-expressing the CtD peptides. Balanced stocks of *UAS-m8SD+UAS-CtD* were crossed to *109-68Gal4* flies (see inset in Fig. 3). We find that co-expression of M8-CtD strongly rescued the reduced eye of *109-68Gal4>UAS-m8SD* flies (Fig. 3b), and significantly restored the hexagonal facet phasing and the positioning of the IOB's (Fig. 3b'). In addition, M8-CtD largely restored the aberrant ommatidial size that was seen throughout the eyes of *109-68Gal4>UAS-m8SD* flies (compare Fig. 3a', b'), although a few areas of perturbation remained (dotted circle in Fig. 3b'). In contrast, co-expression of M8SD-CtD did not rescue the reduced and rough eye of *109-68Gal4>UAS-m8SD* flies (Fig. 3c), and neither did it rescue the aberrant ommatidial size, IOB patterning loss, and occasional 'fused' ommatidia that are seen in M8SD eyes (Fig. 3a', c').

To quantitatively compare the effects of M8-CtD and M8SD-CtD on the reduced eye, we determined facet numbers in ≥15 flies of the relevant genotypes, an approach previously used by others and us (Kahali et al., 2009; Kunttas-Tatli et al., 2009; Shepard et al., 1989). Akin to wild type flies (not shown), *109-68Gal4/+* flies display ~779±20 facets, whereas *109-68Gal4/+ UAS-m8SD/+* flies display ~411±26 facets (Fig. 3d); the latter number served as the baseline. We find that co-expression of *UAS-m8-CtD* elicited facet numbers (~650-670) that were significantly higher than the baseline when tested with two independent insertions (lines S4 and S5, Fig. 3d). In contrast, co-expression of *UAS-m8SD-CtD* elicited facet numbers (~430) that were indistinguishable from the baseline in the two independent insertions that were examined (lines D4 and D5, Fig. 3d), suggesting that the phosphomimetic CtD was impaired for inhibitory activity in '*trans*'.

In addition to the reduced eye, *109-68Gal4>UAS-m8SD* flies display a strong loss of the four scutellar MC's (Fig. 3e), because this enhancer trap is active in the bristle PNC's (Kahali et al., 2009; Powell et al., 2004). We, therefore, assessed whether this neural defect is modulated by the CtD-peptides. Indeed, co-expression of M8-CtD restored ~2 MC's/scutellum, whereas the effects of M8SD-CtD appeared markedly weaker (Fig. 3e). These effects were not line-specific, as they were recapitulated with two independent insertions of *UAS-m8-CtD* or *UAS-m8SD-CtD* (see Fig. 3e). It therefore appears that the eye and bristle loss of ectopic M8SD are more potently suppressed by M8-CtD, as compared to M8SD-CtD. Since the reduced eye of M8SD is insensitive to CK2- or E(spl)-dosage (Kahali and Bidwai, unpublished), the possibility arises that suppression by M8-CtD involves DN-effects mediated by its interaction with ectopically expressed M8SD.

M8-CtD more strongly suppresses the bristle defects of ectopic M8.

We sought to further assess the differences in activity of M8-CtD versus M8SD-CtD. We have recently found that loss of the MC's elicited by ectopically expressed wild type M8

is strongly rescued by co-expression of a *UAS-CK2α-RNAi* construct or *UAS-Tik*, a CK2-DN (Bose et al., 2006; Kahali et al., 2009). These results suggested that phosphorylation also regulates antagonism of ASC by M8 during SOP selection (Fig. 1a). Crosses were conducted as before (inset in Fig. 4) to test whether the CtD has similar effects on the MC loss of ectopic M8, and if a difference between the two phospho-forms is evidenced. This appears to be the case. In contrast to the almost complete loss of the four scutellar MC's in *G455.2>UAS-m8* flies (Fig. 4a, d), co-expression of M8-CtD restored ~2-3 MC's/scutellum (Fig. 4b, d). Often, socket cells and misshapen MC's were observed (see dotted circles in Fig. 4b), as have previously been reported upon *G455.2* driven expression of an antimorphic variant of M8 (Giebel and Campos-Ortega, 1997). Importantly, and in contrast, co-expression of M8SD-CtD did not restore scutellar MC's of *G455.2>UAS-m8* flies (Fig. 4c, d). This difference in activity of M8-CtD versus M8SD-CtD was observed with multiple independent insertions (Fig. 4d).

It therefore appears that the phosphorylation status of Ser^{159} does influence the DN-activity of the CtD during eye and MC development, i.e., M8-CtD suppresses the neural defects of ectopic M8SD or M8 more strongly than does M8SD-CtD. The stronger rescue by M8-CtD is unlikely to involve sequestration of Gro *per se*, given its equivalent interactions with both CtD-variants (Fig. 1d). Moreover, these effects are unlikely to involve competition between two *UAS*-constructs for (rate) limiting amounts of Gal4 produced either by the *G455.2* or *109-68Gal4* enhancer traps, because no modulation of the neural defects of ectopic M8SD or M8 is seen upon co-expression of a *UAS-LacZ* construct (data not shown and Kahali et al., 2009).

M8-CtD, but not M8SD-CtD, rescues the severely reduced eye of *N^{spl}/Y; E(spl)D/+* flies.

The severely reduced eye of *N^{spl}/Y; E(spl)D/+* (Fig. 5a) has provided important mechanistic insights into repression by M8 (Giebel and Campos-Ortega, 1997; Kahali et al.,

2009; Karandikar et al., 2004; Nagel et al., 1999; Tietze et al., 1992). Given the ability of M8-CtD to negate repression by ectopic M8SD or M8, we next tested whether this peptide would display similar activity in an *E(spl)D* mutant background.

Expression was driven immediately anterior to the MF with h^{H10}*Gal4*, as has previously been used for analysis of the activity of E(spl)-variants during eye development (Kahali et al., 2009; Ligoxygakis et al., 1998; Nagel et al., 1999). Indeed, the severe reduced eye of N^{spl}*/Y; E(spl)D/+* flies was partially rescued upon overexpression of M8-CtD (Fig. 5b). As previously reported (Karandikar et al., 2004; Nagel et al., 1999) the residual eye field of N^{spl}*/Y; E(spl)D/+* flies (Fig. 5a) is highly disorganized, and the few remaining facets are not juxtaposed; the clustering of the IOB's might well reflect facet loss. Expression of M8-CtD not only increased eye size (facet numbers), but also restored patterning of the facets (Fig. 5b'). Examination of a higher magnification (Fig. 5b') indicates that ommatidia at the posterior margin are closely juxtaposed and patterned akin to that in the wild type (not shown). This patterning is, however, lost towards the anterior margin of the residual eye (see arrow in Fig. 5b'), and presumably reflects more posterior expressivity of h^{H10}*Gal4*, as has been reported (Ligoxygakis et al., 1998). Analogous to our results with ectopic M8SD or M8 (see above), the reduced eye of N^{spl}*/Y; E(spl)D/+* flies was unaffected by h^{H10}*Gal4*-mediated expression of M8SD-CtD (Fig. 5c, c'). As controls, we tested and found that N^{spl}*/Y; E(spl)D/*h^{H10}*Gal4* flies, or N^{spl}*/Y; UAS-CtD/+; E(spl)D/+* flies, all displayed a severely reduced eye akin to that in N^{spl}*/Y; E(spl)D/+* flies (data not shown). These results indicate that the h^{H10}*Gal4* enhancer trap does not, on its own, rescue the reduced eye of N^{spl}*/Y; E(spl)D/+*, and that expression of the CtD is required. Given the inability of ectopic M8SD-CtD to increase eye size, it appears that rescue is specific to M8-CtD.

We also quantified rescue by determining facet numbers. N^{spl}*/Y; E(spl)D/+* flies typically display ~5-15 ommatidia, and this number was used as the baseline (see Fig. 5e,

f). Consistent with the adult eye (Fig. 5c), overexpression of M8SD-CtD did not increase facet numbers to any significant degree in three lines (D1, D2 and D3) that were tested (Fig. 5f). In contrast, expression of M8-CtD resulted in facet numbers (~150-170) that were significantly higher than in *N^{spl}/Y; E(spl)D/+* flies (Fig. 5e). Importantly, levels of facet restoration were quantitatively similar upon expression of three (second chromosome) insertions of *UAS-m8-CtD* (S1, S2 and S3, Fig. 5e).

Rescue of the *N^{spl}/Y; E(spl)D/+* reduced eye requires MF-specific expression of M8-CtD.

We next assessed whether the effects of M8-CtD display MF-specificity. During the onset of eye development, R8 specification occurs in a spatially precise manner within the MF, a moving wave of differentiation that sweeps across the third instar eye imaginal disc (Wolff and Ready, 1991). While R8 specification initiates at the anterior margin of the MF, the selection of single phase-shifted R8's is completed at its posterior margin. In this regard, it is the specification of R8 cells that is severely blocked in *N^{spl}/Y; E(spl)D/+* flies (Nagel et al., 1999). This block in R8 formation precipitates loss of the eye field because R8 photoreceptors are singularly responsible for recruitment of all secondary retinal cell types (reviewed in Frankfort and Mardon, 2002; Hsiung and Moses, 2002). Given this, we reasoned that if rescue involved inhibitory activity of M8-CtD (in *'trans'*) against M8*, expression of this peptide posterior to the MF should be without effect. For this, we used *gmrGal4*, a driver that is active in all cells posterior to the MF (see inset in Fig. 5g, Bessa et al., 2002). Consistent with our prediction, expression of M8-CtD with *gmrGal4* did not rescue the severe reduced eye of *N^{spl}/Y; E(spl)D/+* flies (Fig. 5d). The absence of rescue is also supported by facet numbers, which upon expression of three independent *UAS-m8-CtD* lines closely mimicked baseline numbers in *N^{spl}/Y; E(spl)D/+* flies (Fig. 5g). The absence of an effect is more striking, given that lines S1, S2 and S3 all elicited rescue when expressed

anterior to the MF with $h^{H10}Gal4$ (Fig. 5b, e). Rescue of the N^{spl}/Y; *E(spl)D/+* retinal defects by M8-CtD, therefore, requires expression in a region of the developing eye where founding R8's are patterned and specified.

Discussion:

Neural patterning requires repression by E(spl) proteins, and it is therefore important to define the underlying mechanisms. Repression has been considered to involve redundant mechanisms, in part, reflecting conserved domains and overlapping phenotypes upon ectopic expression of individual E(spl) members. As a result, repression has been thought to reflect their expression and ensuing accumulation in the non-SOP's/R8 cells in response to inhibitory N signaling. Accumulating evidence suggests that this model might obscure mechanistic diversity of these proteins. This is best exemplified during R8 selection, where ectopic expression of wild type Mδ (but not M8 or Mγ) affects eye development, a finding interpreted to reflect 'qualitative' differences between these members (Ligoxygakis et al., 1998). A definitive role for M8 in R8 patterning is, however, illustrated by the dominant effects of the *E(spl)D* mutation, and the finding that a similar outcome is elicited by M8SD, a phosphomimetic variant. These studies raise the possibility that functional diversity involves differential phosphorylation. We have focused on the M8 protein because the *E(spl)D* mutation provides an excellent framework for structure/function studies to more clearly define the mechanisms by which this protein mediates neural repression. As part of this effort, we have investigated the role of phosphorylation of the CtD in autoinhibition. The studies we present here provide more direct evidence for this layer of regulation and, in addition, suggest that diversity of the CtD's might underlie the 'qualitative' differences ascribed to by Baker and co-workers (Ligoxygakis et al., 1998).

Our studies more directly test the autoinhibition model (see Fig. 1) in two relevant developmental contexts, the eye and the bristle, where endogenous E(spl) mediates R8/SOP selection. In support of this model, we provide multiple lines of evidence that M8-CtD displays stronger (inhibitory) activity than does the phosphomimetic variant M8SD-CtD. This difference in the activity of the CtD's is recapitulated with multiple independent insertions, and is best seen with their ability to rescue the neural defects associated with ectopic expression of M8SD or M8 (Fig. 3, 4). Although expression levels of the CtD peptides have not been determined, our findings collectively suggest that the differences we observe likely reflect inhibitory activity engendered by interaction of M8-CtD with M8SD or with M8 (in its phosphorylated state) in vivo.

Rescue by M8-CtD is unlikely to reflect sequestration of Gro, which is not rate limiting for N signaling (Nagel and Preiss, 1999). Moreover, suppression by M8-CtD is unlikely to involve competition for, or sequestration of, endogenous CK2. The interaction of CK2 with M8 or M8-CtD is disrupted upon phosphorylation (Karandikar et al., 2004 and Kahali and Bidwai unpublished), and while halved CK2 dosage is sufficient for proper N signaling, its further knockdown leads to supernumerary ('twinned') SOP's/R8's, and to rough eyes due to impaired lateral inhibition (Bose et al., 2006). Consistent with the possibility that (endogenous) CK2 levels are not rate limiting for lateral inhibition, the reduced eye of M8SD is not suppressed by decreased CK2 levels or E(spl)-dosage (Kahali, unpublished). In accordance with the autoinhibition model, the DN-activity of the M8-CtD peptide likely reflects its ability to interact with and neutralize repression by endogenous or ectopic E(spl). An alternative possibility is that the DN-effects of the CtD, instead, reflect its interaction with Ato/ASC. Since interactions between E(spl) and Ato/ASC underlie lateral inhibition, binding of CtD to Ato/ASC could also prevent M8 or M8SD from mediating neural repression. A number of lines of evidence argue against this alternative possibility. First,

M8SD and M8* bind Ato with near identical strength, suggesting that the CtD does not contribute to the Ato interaction. Second, the ability of M8* to interact with Ato is, in fact, abolished by removal of the Orange domain. This is best exemplified by *E(spl)*^{BE25}, a revertant allele of *E(spl)D* that lacks Orange and does not elicit a reduced eye in the presence of *N*^{spl}(Nagel et al., 1999). Third, if the M8SD-Ato interaction were to involve the CtD, the DN-effects would have been seen with M8SD-CtD, rather than M8-CtD. Our findings that only M8-CtD displays potent DN-effects do not support this alternative possibility. The most parsimonious interpretation is that binding of M8-CtD to ectopic M8SD, or to the phosphorylated state of ectopic M8 (M8^{PO4}, the non-inhibited state), attenuates repression.

The rescue of the severely reduced eye of *N*^{spl}*/Y; E(spl)D/+* by M8-CtD is the first such example of modulation by an ectopically expressed E(spl) sub-domain. In this case, M8-CtD rescues the reduced eye, albeit partially, and significantly restores ommatidial numbers and patterning, as well as the specification of the IOB's at alternating positions of the ommatidial lattice. Importantly, no such effects are seen when M8-CtD is expressed posterior to the MF (with *gmrGal4*), and are not observed with the phosphomimetic M8SD-CtD peptide that is predicted to bind with reduced strength (Fig. 5).

How might one interpret the rescue of *N*^{spl}*/Y; E(spl)D/+*? During development of the founding R8 photoreceptors, N signaling occurs in a biphasic manner in the MF of the developing third instar eye disc (reviewed in Baonza and Freeman, 2001). At the anterior margin of the MF, this mediates *ato* expression, which is subject to a positive feedback loop (Sun et al., 1998), whereas in the MF it drives expression of *E(spl)* enabling refinement of single R8 cells from the PNC's. *N*^{spl} renders R8 precursors hypersensitive to inhibitory N signaling, and thus impairs R8 specification (Li et al., 2003). The loss of differentiated R8's, in turn, impairs expression of Hedgehog and Decapentaplegic, whose activities are

necessary for *ato* expression (Li et al., 2003). In this sensitized background, M8* (*E(spl)D*) further decreases *ato* expression, by impairing the positive feedback loop (Kahali et al., 2009). As a result, Ato levels drop below a threshold necessary for conferring the R8 fate, and results in a loss of the eye field. Given its effects on Ato, the retinal defects of *E(spl)D* are enhanced by mutations in *ato* or its heteromeric partner, *daughterless* (*da*), whereas they are suppressed by mutations in *Delta* (*Dl*), which would reduce inhibitory N signaling in R8 precursors (Nagel et al., 1999; Shepard et al., 1989).

It would, therefore, seem to be the case that M8-CtD binds to non-autoinhibited M8* (Fig. 5), which would attenuate its interactions with Ato. This would attenuate that ability of M8* to impair the *ato* feedback loop, and consequently Ato would rise to a level sufficient to confer the R8 fate, which would increase eye size (ommatidial numbers). Our finding that the antimorphic activity of M8-CtD is manifest only when expressed anterior to the MF, but not posterior to it (Fig. 5), is consistent with the findings that *E(spl)D* effects are MF- and R8-specific (discussed in Kahali et al., 2009). Therefore, the possibility arises that the inability of M8SD-CtD to rescue the eye defects of N^{spl}*/Y; E(spl)D/+* reflects a reduced capacity, or perhaps an inability, to bind M8*. Under these conditions, M8* retains its ability to bind to Ato and block eye development. Collectively, the effects we see (Figs. 2-5) are consistent with autoinhibition.

The domains/motifs that are highly conserved in all E(spl) proteins are the b/HLH and Orange and WRPW. While the HLH domain appears to mediate homo/hetero-dimerization, Orange is thought to be the specificity determinant (Dawson et al., 1995). In this regard, autoinhibition by the CtD could involve interactions with either domain (Fig. 1a). If this involved the HLH domain, the autoinhibited state of M8 is predicted to be a monomer, a conformation that is non-permissive for repression (discussed in Giebel and Campos-Ortega, 1997). In the yeast two-hybrid assay, dimerization of M8 does appears weaker than

that of other E(spl) proteins (Alifragis et al., 1997; Nagel et al., 1999), but it is not known if this weak interaction reflects the native state or is a consequence of misfolding. Alternatively, the CtD interacts with Orange. In this case, the CtD, in a phosphorylation dependent manner, would regulate antagonism of ASC/Ato (Fig. 1a). The suppression of neural defects of ectopic M8 or M8SD, and the rescue of the reduced eye of N^{spl}/Y; *E(spl)D/+* (by ectopic M8-CtD) would be consistent with its interactions with either HLH or Orange. We attempted, but were unable to detect interactions between M8-CtD and either M8* or M8SD (data not shown), even though these proteins are expressed in yeast (Fig. 1d, and data not shown). Given the small size of the CtD peptides, the possibility remains that expression as a (two hybrid) fusion impedes protein-interactions. Alternative approaches on native proteins and isolated CtD peptides, such as chemical cross-linking and peptide mapping, or surface plasmon resonance, will be needed to identify the interaction site/interface.

Autoinhibition by the CtD, which represents a novel proposition in the regulation of repression by E(spl)-M8, is an established mechanism regulating other proteins. Well described in protein kinases, e.g., the MAPK's and cyclin-dependent protein kinases (CDK's), autoinhibition and phosphorylation are increasingly found to regulate transcription factors (reviewed in Gardner and Montminy, 2005; Schlessinger, 2003; Smock and Gierasch, 2009; Tokuriki and Tawfik, 2009). An excellent example is IRF-3 (Interferon Regulatory Factor-3). Crystal structure of IRF-3 shows that a highly disordered region, the autoinhibitory peptide, binds to a two-helix bundle preventing dimerization (Qin et al., 2003). Phosphorylation of this peptide elicits displacement, which then permits dimerization and transcriptional activities. It has, therefore, been proposed that autoinhibition via a 'reversible intra-molecular latch' is dynamically coupled to signaling (Smock and Gierasch, 2009). Such regulation influences the activities of diverse transcription factors such as Vav, Ets,

Fos, Myc, and p53 (reviewed in Garza et al., 2009). More recently, it has been suggested that such regulatory regions, often the sites for phosphorylation, are 'intrinsically disordered' (ID), and that the co-localization of these features underlies functional diversity (Smock and Gierasch, 2009; Tokuriki and Tawfik, 2009). Consistent with this, these regions of ID have evolved rapidly, are enriched in Ser residues, and often contain motifs for phosphorylation as well as protein interactions (Collins, 2009).

Structure and sequence analyses indicate that such ID regions exhibit a preference for certain amino acids (reviewed in Dunker et al., 2008; Tompa, 2002). Based on these data, computational approaches have been developed to predict their presence (reviewed in Lee et al., 2007). We reasoned that in the case of the E(spl) proteins, if regions of sequence divergence, phosphorylation, and ID-propensity co-localize to the CtD, it would further implicate this sub-domain in the regulation of repression and in non-redundancy. Aside from small differences at the N-terminus, the CtD significantly contributes to the length/sequence heterogeneity of E(spl) members (Fig. 6a). This is best illustrated by the conservation of the SPxS----**S**DxE/D motif. The former represents a putative MAPK site, while the latter is the known CK2 recognition/phosphorylation site in M8/5/7 (Fig. 6a). A closer examination reveals the presence of the SPxS motif in Mγ that is separated from a CK2 phosphorylation site **S**^{195}EDE (Fig. 6a, b). The latter site, which fully conforms to the consensus for CK2, i.e., S/T-D/E-x-D/E (Kuenzel and Krebs, 1985), has remained invariant in Mγ homologues through Drosophila evolution (see alignment in Fig. 6b). Moreover, the replacement and/or insertions upstream of Ser^{195} in some Mγ members do not affect the consensus requirements for CK2. Evolutionary principles would suggest that this conservation is, therefore, of consequence. It will be of interest to determine if Mγ is modified at Ser^{195} by CK2, and whether its replacement with Asp generates an active repressor that elicits a reduced eye, analogous to M8SD.

Given that M8, Mγ and Mδ are the only members expressed in the MF, we assessed whether the CK2 site localizes to an ID-region. This appears to also be the case for M8 and Mγ (Fig. 6b). In contrast, Mδ, the only member that blocks eye development when over-expressed (Ligoxygakis et al., 1998), is predicted to be largely globular and lacks an ID-region or CK2 phosphorylation site. As shown in Fig. 6b, the other ID-regions localize to the sequence between HLH and Orange (in Mγ and Mδ), or the loop connecting the two helices of Orange (in M8). These loops might, however, be structurally constrained by their positioning between two helices. In the case of Hes6, the mammalian homolog of Drosophila E(spl), an ID region and CK2 site also co-localize (not shown), suggesting that the stimulatory effects of phosphorylation on Hes6 activity (Gratton et al., 2003) may reflect a similar regulation.

The possibility thus arises that, as with M8, repression by Mγ might also be regulated by phosphorylation, whereas Mδ is independent. If so, the 'qualitative' differences between these three E(spl) members, proposed by Baker and co-workers (Ligoxygakis et al., 1998), may reflect diversity of the CtD's, and their differential phosphorylation in an isoform-dependent manner. Future studies on the region contacted by the non-phosphorylated CtD of M8, the relevance of this mechanisms to other E(spl) members, and analysis of chimeric proteins will be required to more fully define the diverse mechanisms underlying repression by this group of bHLH proteins.

Materials and Methods:

Construction of CtD variants:

The region of the cDNA's encoding residues 123-179 of M8 and M8SD were amplified by PCR using custom primers, and contain BamH1 and Xho1 sites 5' and 3' to the open reading frame, respectively. The PCR products were verified by sequencing.

Phosphorylation and protein interactions:

Phosphorylations were conducted using CK2 holoenzyme purified to homogeneity from Drosophila embryos (Karandikar et al., 2005). Bacterially expressed CtD peptides were purified as fusions with glutathione-S-transferase (GST). Following purification, the GST-fusion proteins were exchanged into storage buffer (50 mM Tris, pH 8.0, 0.5 mM EDTA, 10% glycerol, 200 mM NaCl, 1mM PMSF) using a Biomax-10K centrifugal filter device (Millipore). Phosphorylations were carried out in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 μM ATP, 5 μCi [γ-^{32}P]-ATP in a total volume of 40 μl, at 25^{o}C for 10 min. GST-fusions (2 μg each) were phosphorylated with 40 ng of the CK2-holoenzyme for 15 min at 25^{0}C, conditions that ensure an assessment of the initial rates (Bidwai et al., 1993). The reactions were terminated with 10 μls of 5x sample buffer, boiled for 5 minutes, and separated on 12% acrylamide gels containing sodium dodecylsulfate. Gels were stained with Coomassie Blue, and exposed to Kodak XAR-5 film.

Interactions with Gro were assessed using a LexA version of the yeast two-hybrid system (Gyuris et al., 1993). LexA-CtD constructs were tested against a fusion of Gro with the activation-domain of protein B42. Interactions were assessed in yeast strain EGY048. *LacZ* reporter activity was determined for at least three independent transformants, each in triplicate, as described. *LacZ* activity was determined using the formula $1000xOD_{420}/(TxVxOD_{600})$, where T is minutes and V is the concentration factor of the assay.

Fly stocks, crosses and phenotypes:

For *in vivo* expression, constructs encoding M8-CtD and M8SD-CtD were cloned into the plasmid pUAST (Brand and Perrimon, 1993). Both constructs are identical in length and 5' and 3' ends, aside from the missense mutation at Ser^{159}. Germ line transformants were generated using a commercial embryo injection facility (BestGene, Inc.). w^{+} progeny were

identified and the location of insertions was determined via crosses to lines harboring chromosomes carrying dominant visible markers. Multiple independent insertions of each construct were used in these studies.

Flies were raised at 24^{o}C on standard Yeast-Glucose medium. The *Gal4* drivers used in these studies are *G455.2*, 1*09-68Gal4*, h^{H10}*Gal4*, and *gmrGal4*. Fly heads were dehydrated by sequential passes through a graded alcohol series (25-50-75-99%) and finally through Hexamethyldisalizane. Heads were mounted on EM stubs, dried for 24 hours, sputter coated with gold, and examined with a JEOL-6400 scanning electron microscope at an accelerating voltage of 10-20 kV. For bristle phenotypes, newly eclosed adults were photographed. For quantitative analysis of the bristle phenotypes, multiple crosses were established (≥triplicates), and adults were scored for bristle artifacts. For quantitative analysis of the eye field, ≥15 adults were photographed, and ommatidial numbers were determined manually. In every case multiple independent insertions of *UAS*-constructs were used.

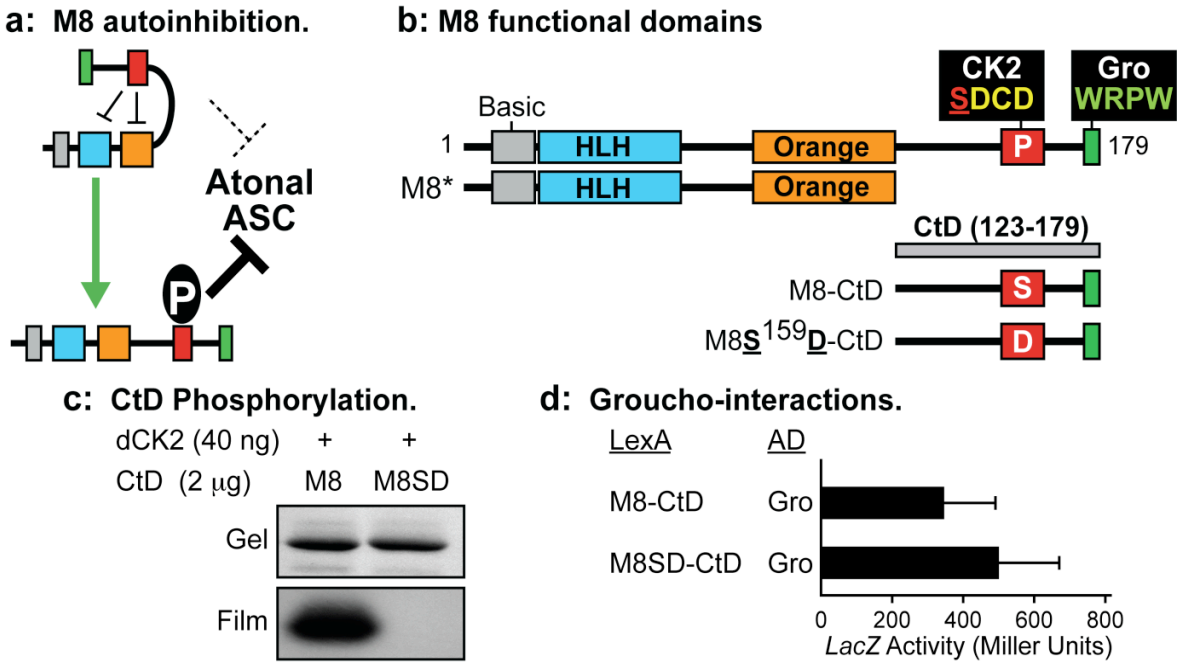

Figure 1. Role of phosphorylation of M8 and characterization of the CtD. (a) The CtD autoinhibits M8, and phosphorylation favors a conformational state permissive for antagonism of ASC/Ato (color of boxes corresponds to those in panel b). (b) Functional domains in M8 and M8*. The C-terminal domain (CtD) peptide encompasses residues 123-179 of full-length M8, and harbors the CK2 phosphorylation and Gro-binding sites. (c). In vitro phosphorylation of GST-CtD fusions by Drosophila CK2 (dCK2). (d) Two hybrid interaction with Gro.

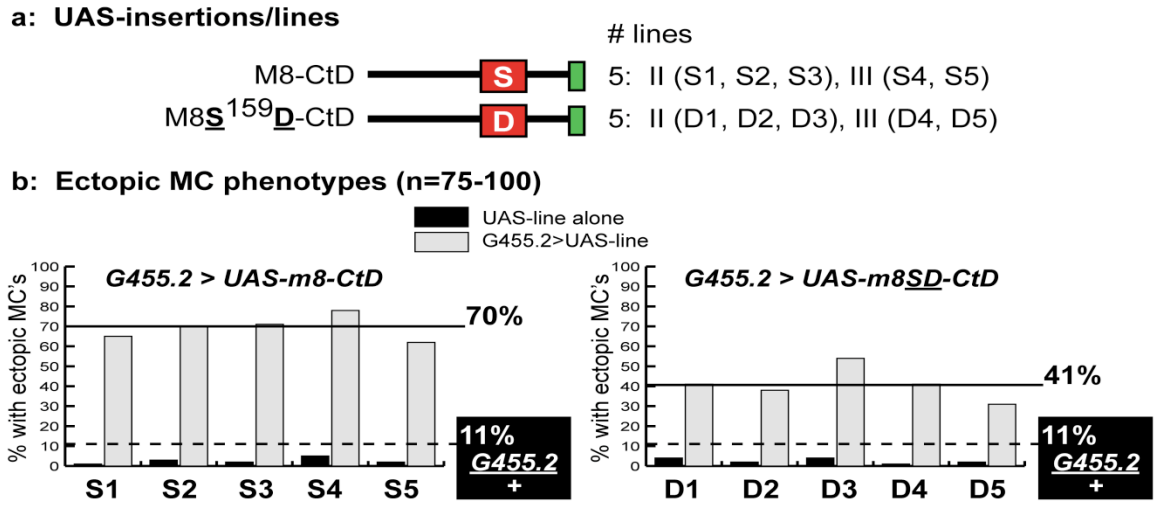

Figure 2. M8-CtD elicits stronger ectopic bristle defects than does M8SD-CtD. Crosses between *G455.2* and *UAS-constructs* were conducted at 24°C. (a) Five independent insertions of *UAS-m8-CtD* (S1-S5) or *UAS-m8SD-CtD* (D1-D5) were analyzed. (b) The penetrance of ectopic scutellar macrochaetes (MC's) in the absence of expression (solid bars) or upon expression with *G455.2* (grey bars). The dashed line denotes the baseline MC defects of *G455.2/+* flies, and the solid line denotes the average MC defect penetrance upon expression of M8-CtD or M8SD-CtD.

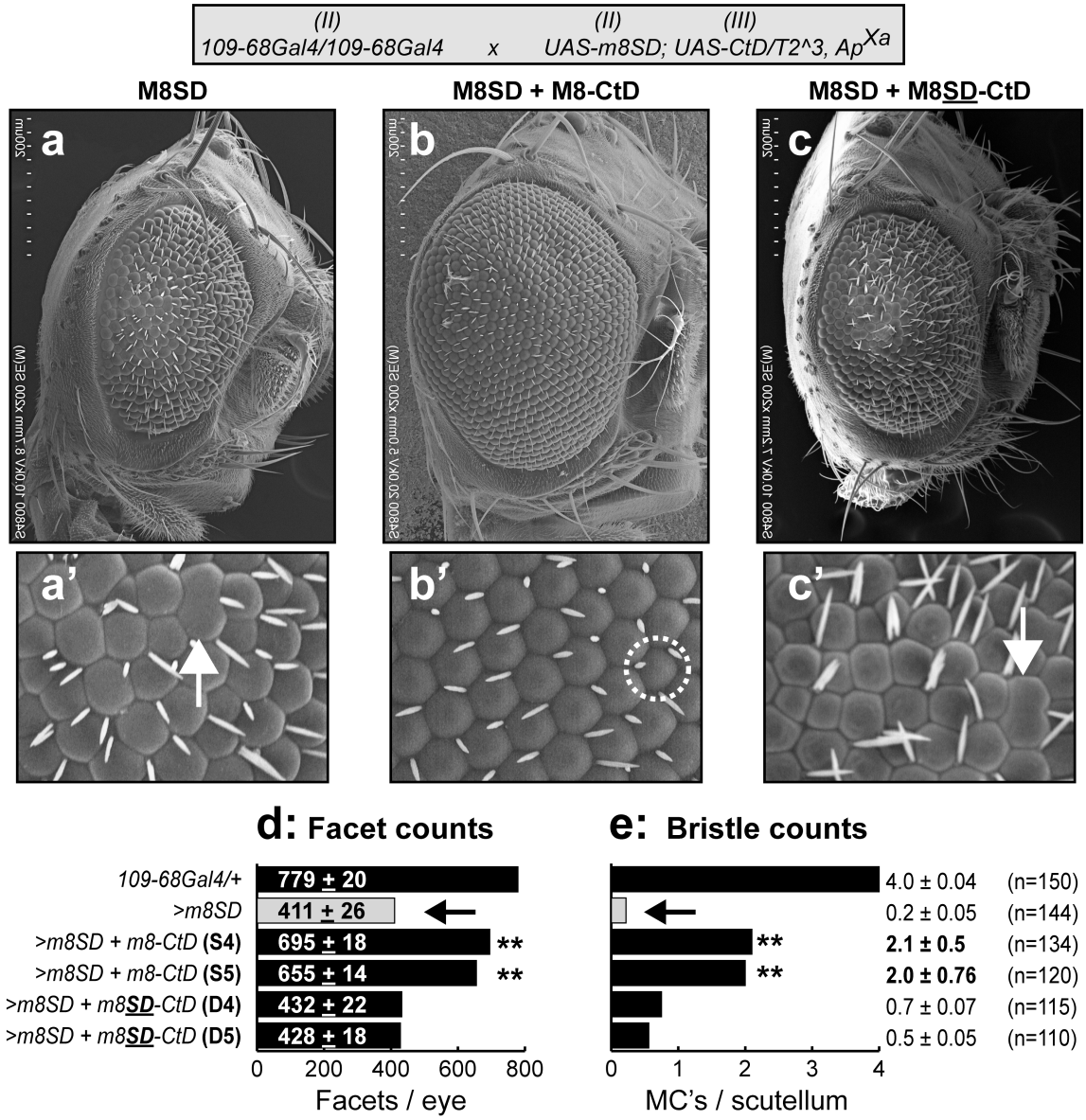

Figure 3. M8-CtD suppresses the eye and bristle defects of ectopic M8SD. Crosses between *109-68Gal4* and *UAS-constructs* were conducted at 24°C. (a-c) Adult eye phenotypes. The proteins expressed are indicated, anterior is to the right, and magnification is 200x. (a'-c') magnifications of regions of the eye in panels a-c; arrows denote fused ommatidia. (d) Rescue of the reduced eye of *109-68Gal4>m8SD* flies (grey bar) upon co-

expression of the CtD's. The *UAS-m8-CtD* and *UAS-m8SD-CtD* lines used are indicated in parenthesis. Asterisk denotes *P*-values <0.001. (e) Suppression of the MC defects of *109-68>m8SD* flies (grey bar) upon co-expression of the CtD's. Numbers to the right denote average scutellar MC counts.

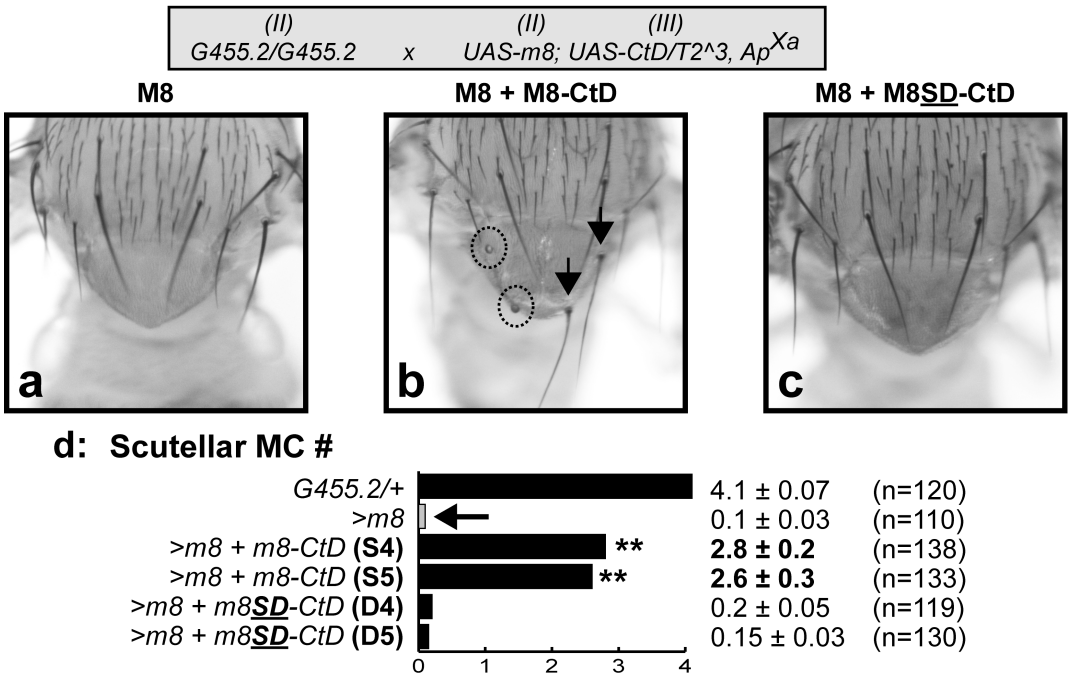

Figure 4. M8-CtD suppresses the MC loss phenotype of ectopic M8. Crosses between *G455.2* and *UAS-constructs* were conducted at 24°C. (a-c) Nota of flies expressing M8 alone (a), M8+M8-CtD (b), and M8+M8SD-CtD (c). Arrows in panel b denote restored scutellar MC's and dotted circles denote socket cells. (d) Suppression of the MC defects of *G455.2>m8* flies (grey bar) upon co-expression of the CtD's. The *UAS-m8-CtD* and *UAS-m8SD-CtD* lines used are indicated in parenthesis. Asterisk denotes *P*-values <0.001. Numbers to the right denote average scutellar MC counts.

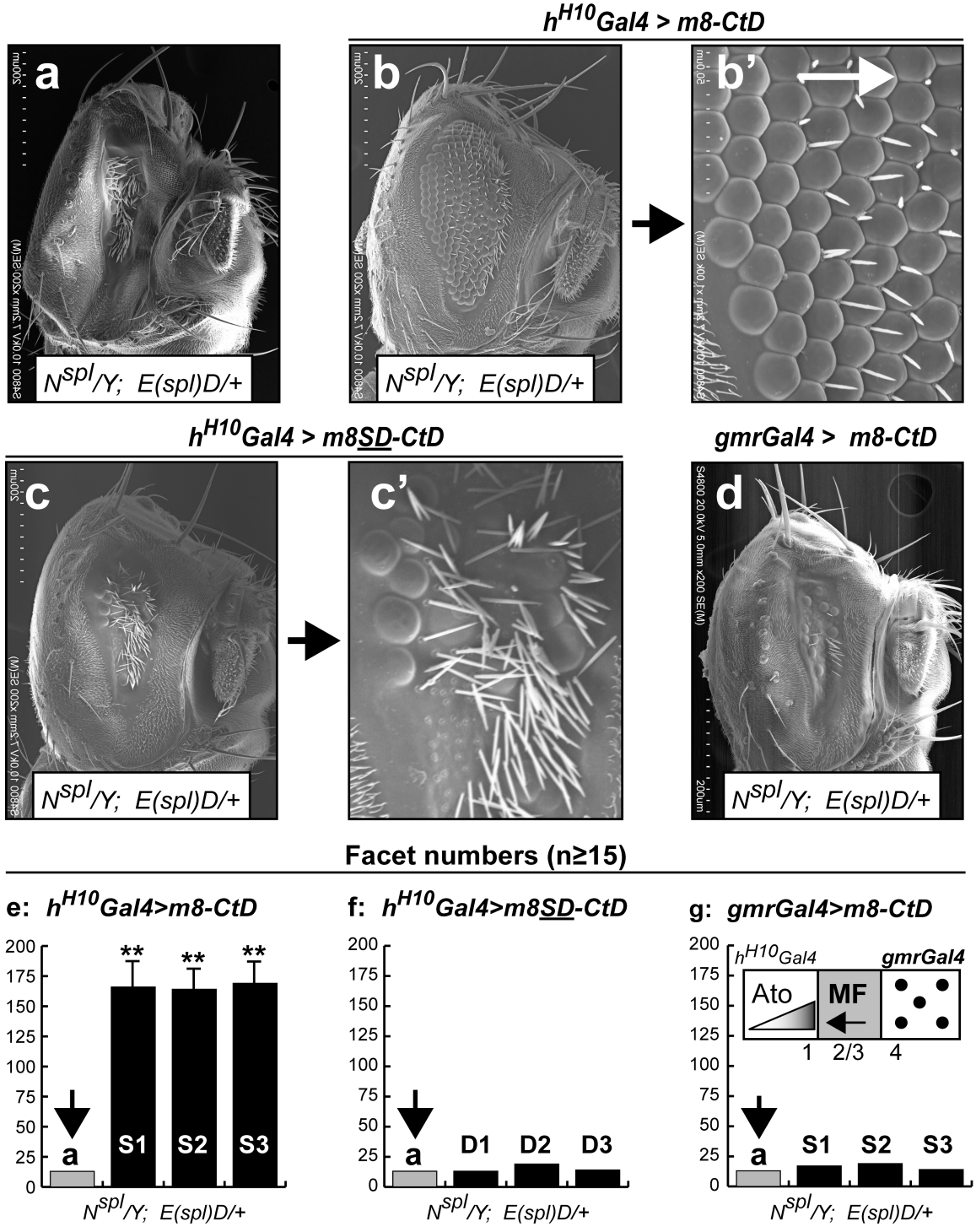

Figure 5. M8-CtD rescues the severely reduced eye of *N^{spl}/Y; E(spl)D/+* flies. (a-d) Adult eye phenotypes. The proteins expressed are indicated, anterior is to the right, and

magnification is 200x. Effects on the reduced eye of N^{spl}/Y; *E(spl)D*/+ flies (a) upon h^{H10}*Gal4* mediated expression of M8-CtD (b), or M8SD-CtD (c). (b', c') Magnification of the eye in panels b and c, respectively. The arrow in b' denotes posterior-to-anterior axis of the eye. (d) Absence of rescue of the reduced eye of N^{spl}/Y; *E(spl)D*/+ upon expression of M8-CtD by *gmrGal4*. (e, f, g) Quantitative analysis of facet numbers of N^{spl}/Y; *E(spl)D*/+ (grey bar and arrow) by h^{H10}*Gal4>m8-CtD* (e), h^{H10}*Gal4>m8SD-CtD* (f), and *gmrGal4>m8-CtD* (g). The *UAS-m8-CtD* and *UAS-m8SD-CtD* lines are indicated, and asterisk denotes *P*-values <0.001. Inset in panel f shows a schematic of the MF, the arrow denotes the MF progression, and the black dots denote R8 cells specified in a phased pattern. While h^{H10}*Gal4* drives expression immediately anterior to the MF, *gmrGal4* drives expression in all cells behind the MF.

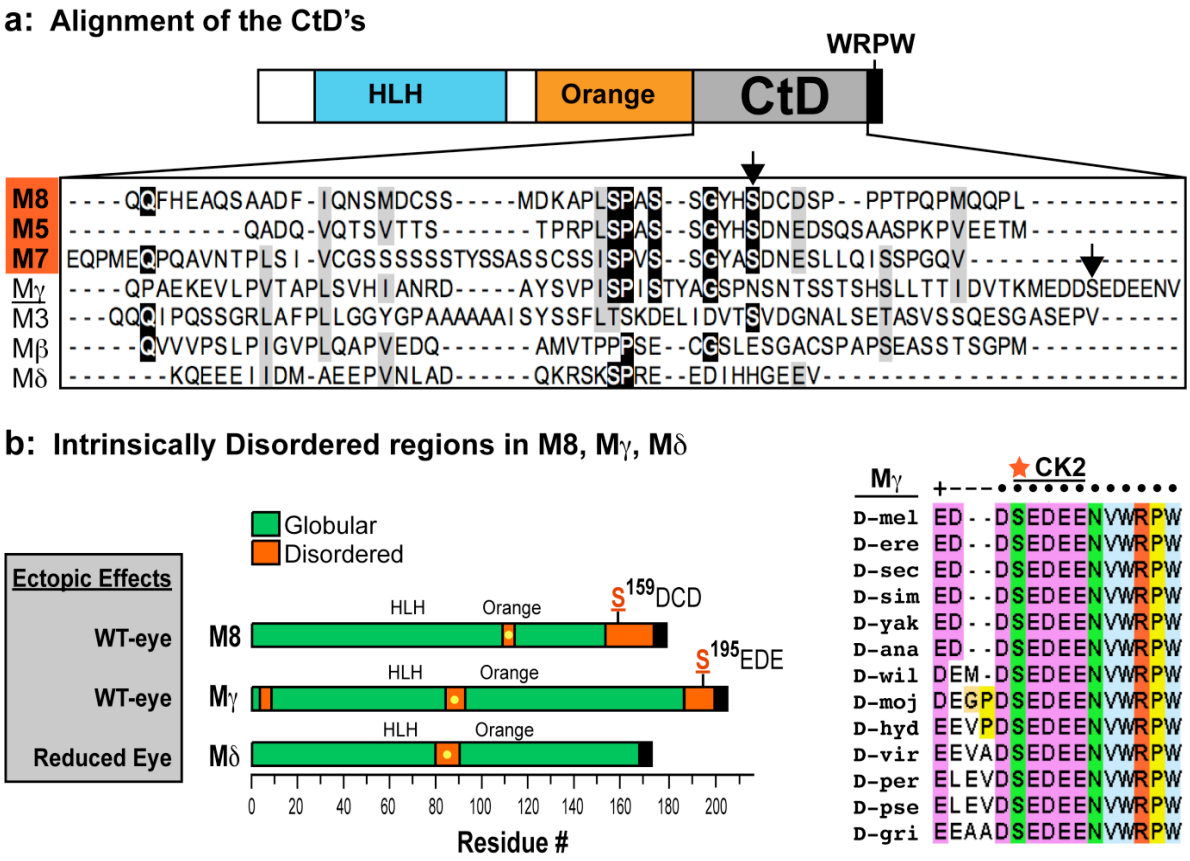

Figure 6. Structure of the CtD. (a) Alignment of the CtD sequences of E(spl) members. Proteins phosphorylated by CK2 are highlighted in red. Mγ is predicted to contain a CK2 consensus site (arrow). (b) GlobPlot-2 (http://globplot.embl.de/) based prediction of globular and ID regions in E(spl) members expressed in the eye. Grey inset shows effects of ectopic expression on eye development. Alignment of Mγ homologs. Abbreviations are; mel, melanogaster; ere, erecta; sec, sechellia; sim, simulans; yak, yakuba; ana, ananassae; wil, willistoni, moj, mojavensis; hyd, hydei; vir, virilis; per, persimilis; pse, pseudoobscura; gri, grimshawi. The Ser of the predicted CK2 site is shown as a star; + denotes conserved substitution; - denotes not conserved/insertion; black dot denotes invariant.

CHAPTER 4

Multisite phosphorylation underlies neural repression by E(spl)M8 in Drosophila.

Abstract:

The ability of E(spl)M8 to antagonize Atonal (Ato) during R8 patterning requires phosphorylation by CK2. The CK2 site resides in a region that conserves a MAPK site, whose contribution to repressor activity was unknown. We report here studies implicating the importance of the MAPK site. Using site-specific variants of M8 harboring Ala/Asp site, we demonstrate that both CK2 and MAPK site modification are necessary for M8 activity against Ato. Consistent with MAPK effects mediated through the EGFR (Drosophila EGF receptor or DER) pathway, we demonstrate that M8 activity is enhanced by gain of function alleles such as *Ellipse (Elp),* whereas it is diminished by halved DER dosage. Our studies indicate that the R8 defects of *Elp* may reflect inappropriate phosphorylation of M8, and that the DER pathway may cooperate with Notch to permit lateral inhibition to occur in a precise stage of retinal development.

Introduction:

Studies involving the compound eye of Drosophila have long served as a model for neuronal specification, and provided important mechanistic insights into how different signaling pathways orchestrate the precise patterning of these cell fates during development. The compound eye of Drosophila is composed of ~800 lights sensing repetitive units (ommatidia). Retinal specification initiates during the third instar larval eye disc, when an apico-basal indentation, termed the morphogenetic furrow (MF), moves across the eye disc towards the anterior margin (Ready et al., 1976). While cells in front of the MF are undifferentiated, those that are posterior to the MF are subject to specification and differentiation in a precise order. R8 photoreceptors are the first to be specified, and these cell types then drive the specification of all the other secondary photoreceptors (R1-7). The Notch signaling pathway is an important determinant during specification of the R8 cells.

R8 specification occurs in the MF, and involves biphasic Notch signaling. In the first phase (anterior to the MF), Notch elicits the expression of Atonal (Ato) to form clusters of equipotent cells, the proneural cluster (PNC's). Later, Notch mediates the selection of a single R8 cell from each PNC, through a process called lateral inhibition (Lehmann et al., 1983; Simpson, 1990). Specifically, the cell destined to become the R8 photoreceptor expresses the Notch ligand Delta (Dl) at a level sufficient to activate inhibitory Notch signaling in the adjacent cells within the PNC (Parks et al., 1995; Simpson et al., 1992), leading to transcription of the *Enhancer of split Complex (E(spl)C* (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). The E(spl) repressors then antagonize Ato activity (Bailey and Posakony, 1995; Ligoxygakis et al., 1998). As a result, Ato activity is sustained only in the R8 cells.

Given the centrality of E(spl) during R8 selection, extensive studies have been directed at uncovering the mechanisms underlying repression of Ato. These studies have been influenced, in part, by the role of E(spl) in the antagonism of activators encoded by ASC during selection of bristle sensory organ precursors (SOP). The ablation of the SOP fate by ectopic expression of any E(spl) member led to the notion that repression may largely reflect E(spl) protein levels (Giebel and Campos-Ortega, 1997; Nakao and Campos-Ortega, 1996; Tata and Hartley, 1995). However, studies in eye have raised doubts whether this is indeed the case. Three E(spl) members, M8, Mγ, and Mδ, are expressed during R8 selection, but only ectopic Mδ elicited loss of the R8's (Ligoxygakis et al., 1999; Ligoxygakis et al., 1998; Nagel et al., 1999). It has, therefore, been proposed that these three E(spl) members are 'qualitatively' different (Ligoxygakis et al., 1999), but the nature of this difference remained unresolved. The central role of M8 during R8 selection is underscored by the eye and R8 defects of the unique dominant allele of *m8*, *E(spl)D.* This allele disrupts retinal histogenesis in the presence of the gain of function *Notch* allele, N^{spl}, a an interaction that led to the original identification of the *E(spl)C* (Welshons, 1956).

The *E(spl)D* allele harbors two lesions; one stabilizes the *m8* transcript, and the second, a nonsense mutation, results in a truncated protein called M8*, which lacks 56 C-terminal residues (Nagel et al., 1999; Tietze et al., 1992). Importantly, ectopic M8*, but not full-length M8, mimics the eye and R8 defects of *E(spl)D*, indicating that the truncation results in a hyperactive protein. To explain this hyperactivity of M8*, the late Dr. Campos-Ortega proposed that the CtD of M8 may regulate M8 repressor activity. The mechanism underlying this hyperactivity remained unclear until it was found that the CtD of M8 is a target for phosphorylation by protein kinase CK2.

CK2 phosphorylates M8 at Ser^{159} in the CtD, and the overexpression of a CK2 phosphomimetic variant ($M8S^{159}D$), was found to elicit loss of R8's due to exacerbated

antagonism with Ato, effects that mimicked those of M8*. To account for the similar outcomes of M8* and M8SD on the R8 cell fate, it was proposed that non-phosphorylated M8 is intramolecularly 'autoinhibited' by its CtD (Fig. 1A). Phosphorylation would relieves inhibition and convert M8 into an active repressor. The truncation in M8* would bypass this regulation, indicating that phosphorylation may be central in regulating M8 activity.

In the case of M8, the CK2 site is located in a region, termed the P-domain that is conserved in all (M8) isoforms through $50x10^6$ years of Drosophila evolution. This region is rich in Ser residues, and harbors consensus sites for phosphorylation by MAPK, CK1 and GSK3. Although, no R8 defects have been described for mutations in CK1 or GSK3, the role of EGFR signaling in R8 specification has long been suspected. This pathway is also called the Drosophila EGF receptor or DER pathway in Drosophila.

The lab of Nicholas Baker has shown that levels of diphosphorylated ERK (dpERK) in the intermediate groups (those undergoing lateral inhibition and R8 selection) are dependent on EGFR pathway activity (Lesokhin et al., 1999; Yang and Baker, 2001). Moreover, DER clones exhibit excess R8 cells at the posterior margin of the MF, an effect also manifested in clones of the downstream effectors of this pathway, such as Ras and Raf. This excess R8 phenotype closely mimics loss of E(spl) or Su(H), both of which are required for the onset of lateral inhibition and R8 selection. It has therefore been proposed that some level of DER pathway activity is "required for lateral inhibition to occur promptly" (Lesokhin et al., 1999).

Other investigators have argued that excess R8's in the DER clones result from secondary effects unrelated to lateral inhibition (Spencer et al., 1998). A careful examination of the data from the Baker laboratory shows that Ato expression initiates normally and cluster formation is unaffected. Since the cells in the MF are cell cycle arrested, the argument of other groups that DER regulates R8 spacing without effects on R8

selection seems incorrect. A direct test for the role of DER in lateral inhibition has not been forthcoming.

The studies we describe here indicate that the MAPK site in M8 is important for repressor activity. We have used site-specific mutants in the CK2 and MAPK sites of M8 and have assessed their activities in the eye. Our studies indicate that modification at both sites appears to be necessary for M8 activity, antagonism of Ato and R8 selection. In this case, CK2 appears to be epistatic to MAPK. Consistent with this, the CK2 phosphomimetic variant of M8 responds in a predictable manner to increased or decreased DER signaling, whereas the variant that is a CK2+MAPK phosphomimic does not. These studies thus indicate that the MAPK site in M8 is responsive to DER signaling. Together, our studies suggest that DER's does play a role in R8 selection, one that involves modification of M8. Our studies support the findings of the Baker lab, and now provide a relevant molecular target for this highly conserved pathway that is reiteratively employed during retinal patterning.

Results:

We have previously reported that expression of a CK2 mimetic variant of M8 with *109-68Gal4* (Fig. 1B) elicits a reduced eye (Fig. 1D), an effect not seen upon its expression with h^{H10}*Gal4* (Fig. 1C). If modification by CK2 were sufficient to override autoinhibition (Fig. 1A), the CK2 mimetic M8S^{4}D protein should have elicited a reduced eye with h^{H10}*Gal4*, as well. Moreover, the severity of the reduced eye of M8S^{4}D should have been higher during the onset of Ato expression as compared to that during the lateral inhibition. We therefore hypothesized that the reduced eye of M8S^{4}D may reflect secondary phosphorylation by kinases that are active only during lateral inhibition (see Fig. 1B). In the case of M8, the CK2 site resides in a region that conserves four Ser residues (Fig. 1E). These Ser residues

numbered 1-4 meet the consensus for MAPK, CK1, GSK3 and CK2, respectively. Of these, human Hes6 conserves the MAPK and CK2 sites. We focused our studies on the contributions of the MAPK site to the eye defects of the CK2 mimetic M8S^{4}D protein.

The reduced eye of M8S^{4}D requires an intact P-domain.

To test if secondary phosphorylation is required for M8 activity, we generated a variant that is mimetic for CK2, but is refractory to secondary phosphorylation (see Fig. 2A). We find that unlike M8S^{4}D, the variant that is refractory for secondary phosphorylation (M8S^{4}D+3A) is significantly impaired (Fig. 2B, C). We also quantified the extent of reduction of the eye field. In this analysis, we determined facet numbers upon expression of multiple *UAS* insertions with *109-68Gal4*. In the case of M8S^{4}D, the eye field was reduced to ~420 facets. In contrast, seven independent insertions of *UAS-m8S^{4}D+3A* exhibited significantly attenuated effects (p-value ≤ 0.001) and ranged from 620-670 facets/eye. While the eye field was significantly restored, it did not approach facet numbers typical of wild type flies (~750 facets/eye; not shown). Thus preventing secondary phosphorylation significantly impairs, but does not abolish M8 activity. The low level of activity of M8S^{4}D+3A may reflect kinetic effects of phosphorylation on conformational changes from the autoinhibited to the active state.

We also stained the eye discs for Senseless (Sens), a marker for differentiated R8's (Nolo et al., 2000), and ELAV, a pan-neuronal marker (Campos et al., 1987). In the case of M8S^{4}D, Sens expression is non-uniform and is not sustained. Consequently, these R8's do not recruit secondary photoreceptors resulting in regions of the eye disc lacking Sens+ELAV clusters (Fig. 2B'). In contrast, M8S^{4}D+3A exhibited more muted effects on R8 specification, differentiation and secondary photoreceptor recruitment, indicating that this protein is an inefficient repressor. Therefore, the reduced eye of M8S^{4}D is likely to reflect its ability to act as a 'sink' for modification by other kinases, which is required for repressor activity.

Secondary phosphorylation enhances M8 activity.

To further assess the importance of these secondary sites, we generated a variant (M8S^{1234}D), which should mimic the fully phosphorylated state (Fig. 3A). Unlike M8S^{4}D, which appears to require secondary phosphorylation for antagonizing Ato, M8S^{1234}D exhibited hyperactivity even when expressed with *h^{H10}Gal4* (Fig. 3B, C). This enhanced activity may reflect a complete bypass of participatory kinases, thereby allowing this tetra phosphorylated variant to elicit a reduced eye. These effects are consistently seen with six independent insertions. A similar analysis with *109-68Gal4* was precluded because expression of M8S^{1234}D with this driver elicited early pupal lethality at 24^{o}C and even at 18^{o}C, where expressivity of this enhancer trap is attenuated (data not shown).

Consistent with the severely reduced eye of M8S^{1234}D, eye discs display severe neural hypoplasia reflecting loss of founding R8's (Fig. 3C'), effects that were dramatically enhanced as compared to those upon expression of M8S^{4}D (Fig. 3B'). These results raised the possibility that control over M8-activity involves multisite phosphorylation.

The MAPK site influences M8 activity.

Given the proposed role of DER signaling in lateral inhibition (see Introduction), we focused on the contribution of the MAPK site (Ser^{151}). To distinguish effects mediated through the CK2 and/or MAPK sites we generated two variants. The first of these, M8S^{1}D, replaces Ser^{151} of the MAPK site with Asp. It is predicted that this variant should be independent of endogenous activated MAPK. The second variant, M8S^{14}D, contains Asp residues at both CK2 and MAPK sites (Fig. 4A). This variant should display activity commensurate with the occupancy of both kinase sites. Multiple *UAS* lines of these variants were generated and their activities were tested during R8 patterning.

We find that M8S^{14}D elicits a reduced eye with a potency that was higher than that of the CK2-mimetic variant M8S^{4}D (Fig. 4B, C). The modest, but reproducible, enhancement

is likely to reflect the possibility that M8S^{4}D is subject to secondary modification at the MAPK site, whereas M8S^{14}D will display the maximal possible activity of a dually phosphorylated protein. A similar analysis with M8S^{1}D (MAPK-mimic alone) did not elicit any eye defects (Fig. 4C). It thus appear to be the case that while necessary, modification at just the MAPK site is insufficient to permit repression of Ato and the R8 fate.

Given our observation that the variant with Asp at all four kinase sites, M8S^{1234}D, elicited severe eye and R8 defects even when expressed with *h^{H10}Gal4,* we next tested if M8S^{14}D could exhibit a similar behavior. We find that unlike the inactivity of M8S^{4}D, expression of M8S^{14}D with *h^{H10}Gal4* led to a reduced eye (4E, G). This enhanced activity of M8S^{14}D might reflect a bypass of phosphorylation by MAPK. No such eye defect was seen when M8 contained an Asp at only the MAPK site, an outcome similar to its inactivity when expressed with *109-68Gal4* (compare Fig. 4C & 4F). This would support the likelihood that modification of both CK2 and MAPK sites is necessary for M8 activity.

The effects of M8S^{4}D are sensitive to altered DER levels.

Studies described in the previous section implicated the MAPK site in regulating M8 activity during lateral inhibition. This raised the possibility that M8 may be a target of activated DER signaling. If so, it is expected that the retinal defects of M8S^{4}D should be sensitive to altered DER dosage or activity, whereas the CK2 and MAPK mimic, M8S^{14}D should be refractory to changes in DER signaling.

For this analysis, we employed two alleles of *egfr,* i.e., *Ellipse (Elp)* and *egfr^{f24}*. *Elp* increases DER signaling, and this gain of function allele perturbs the selection of R8 cells from the preclusters. Consequently, *Elp* leads to a weak loss of R8 cells that results in a slight reduction of the eye field, but the mispatterning of the R8 cells leads to a uniformly rough eye (Baker and Rubin, 1989; Baker and Rubin, 1992; Lesokhin et al., 1999). In

contrast, *egfr*^{f24} is a loss of function (amorphic) allele, which does not perturb the eye in the heterozygous state. Both alleles are lethal when homozygous.

If the DER pathway, indeed, regulates the activity of M8, then a decrease in its levels should attenuate MAPK activity, and lead to hypophosphorylation of M8 at its MAPK site. If so, the reduced eye of M8S^{4}D should be suppressed (rescued) in an *egfr*^{f24}/+ background. We find that this is indeed the case (compare Fig. 5B and 5C). Moreover, the facet counts indicate that halved DER dosage significantly mitigates the reduced eye of M8S^{4}D (Fig. 5D). In contrast, halved DER dosage had no effect on the reduced eye of the CK2 and MAPK mimic M8S^{14}D, given that this variant should be refractory to DER signaling. These observations suggest that the conserved MAPK site does mediate the responsiveness of M8 to altered DER dosage.

Given our observation that M8S^{14}D exhibited eye defects when expressed with *h*^{H10}*Gal4,* we next sought to assess if the CK2 mimic M8S^{4}D could mimic such behavior in a hyperactive DER background (*Elp/+).* We find that expression of M8S^{4}D with *h*^{H10}*Gal4*, indeed, elicits a reduced eye in an *Elp/+* background (Fig. 6C). Moreover, facet counts indicate that the severity of the reduced eye of M8S^{4}D in the presence of *Elp* is comparable to that exhibited by M8S^{14}D in a background with normal DER signaling (compare Fig. 4G and 6F). As observed with halved DER dosage, the reduced eye of the CK2_MAPK mimic M8S^{14}D, was not enhanced in an *Elp/+* background (compare Fig, 6E, F). These observations suggest that the reduced eye of M8S^{4}D in an *Elp/+* background likely reflects the ability of this protein to act as a 'sink' for modification by activated MAPK. Collectively, these results suggest that M8 is a target of the activated DER pathway. Based on the in vivo effects of M8 variants and their response to altered DER signaling, we conclude that DER and Notch cooperate to regulate M8 activity during lateral inhibition.

Discussion:

The activities of E(spl) proteins are vital for mediating the effects of inhibitory Notch signaling, i.e., lateral inhibition. In this context, it has previously been shown that the activity of E(spl)M8 is a regulated process, one that involves phosphorylation of M8 by protein kinase CK2 (Karandikar et al., 2004). The CK2 site in M8 resides in a region that contains a number of Ser residues, some of which are also conserved in the mammalian homolog Hes6 (Fig. 1E). Given the importance of the CK2 site to M8 activity against Ato, the possibility remained that the P-domain is subjected to multisite phosphorylation, and if so may involve the activities of additional protein kinases; MAPK, CK1 and GSK3 (Sgg). This finding raises the possibility that the activity of M8 is an outcome of multisite/hierarchical phosphorylation.

Using site-specific variants of M8, we have characterized the contributions of these secondary phosphorylation sites. Through multiple lines of evidence, we demonstrate that all four kinase sites potentiate M8 activity. Moreover, these studies support our earlier proposal that the strong antagonism of Ato and elicitation of reduced eye by the CK2-phosphomimetic variant M8S^{4}D likely reflects its ability to acts as a 'sink' (Kahali et al., 2009). During R8 selection, these spatially restricted signals may act as a multiple independent regulatory steps that exert control over M8 activity to prevent precocious onset of repression. In this report, we have also uncovered a novel interaction between DER and M8. The studies we describe here indicate that DER signaling cooperates with Notch during lateral inhibition to select a single R8 photoreceptor from an equivalence group.

The role of DER is well established during Drosophila development. Spitz (Spi) is the primary DER activating ligand that plays a critical role during Drosophila eye development (Freeman, 1994b). In addition, the inhibitory ligand Argos (Aos) is produced in response to DER signaling, and functions through a negative feedback loop to fine tune

DER signaling (Freeman, 1994a; Freeman et al., 1992). *spitz* encodes a membrane bound proteins that undergo cleavage by Rhomboid and Star to generate the secreted form of Spi. Secreted Spi then diffuses and binds to the extracellular domain of DER, leading to the activation of the Ras/Raf/MAPK cascade (Sturtevant et al., 1993). In Drosophila, this leads to the activation of the transcriptional activator *pointed* (*pnt),* and repression of *yan* (O'Neill et al., 1994). Target genes of DER pathway also include *argos* that antagonizes DER signaling by directly binding to Spi.

In various developmental contexts, DER and Notch signaling antagonize each other, with DER, generally, promoting a particular cell fate, and Notch inhibiting it (Sundaram, 2005). As such, many members of the DER pathway are found to be expressed in the same developmental stage with Notch. Accordingly, DER alleles have been identified in screens for modifiers of Notch (Verheyen et al., 1996; Weber et al., 2003). Antagonism of Notch and DER pathway is also evident during Drosophila photoreceptor (R1-R6) differentiation. In this context, Notch and Su(H) positively regulate the expression of the repressor, *yan* (Rohrbaugh et al., 2002). In contrast, DER signaling negatively regulates *yan* and positively regulates *pnt* expression. Although, Notch and DER antagonize each other in varied developmental contexts, they do act cooperatively in the specification of R7 and the cone cell fates (Flores et al., 2000; Nagaraj and Banerjee, 2004). The role of DER and its interaction with Notch is well documented during the differentiation of different cell fates in eye development; however, its precise role in R8 specification is not clear.

A potential involvement of DER in the specification of R8 photoreceptors has long been suspected (see introduction). Critical insights came from the analysis of the gain of function *DER* allele, *Elp. Elp* affects R8 selection, resulting in loss of R8 cells (Baker and Rubin, 1989; Lesokhin et al., 1999; Yang and Baker, 2001). Moreover, *Elp* also enhances the eye defects of N^{spl}, a gain of function *Notch* allele (Baker and Rubin, 1992). N^{spl} renders

R8 precursor sensitive to inhibitory Notch signaling, and consequently, leads to loss of R8 cells. As such, N^{spl} represents a hyperactive E(spl) background (Li et al., 2003). Accordingly, a decrease in E(spl) dosage can rescue the N^{spl} eye defects (Parks et al., 1995). Given the observation that *Elp* exacerbates N^{spl} eye defects, underscores the likelihood that DER is indeed active at some levels in intermediate groups. However, the target of this pathway during lateral inhibition remained unknown.

Previous studies have shown that the *Elp* phenotype depends on *aos* to mediate its effects (Lesokhin et al., 1999). In this case, high amount DER is proposed to activate Aos, which in turn, antagonizes Ato. This proposal is supported by the observation that ectopic expression of Aos leads to loss of Ato expression, and consequently, elicits aberrant R8 spacing (Spencer et al., 1998). These observations led to the suggestion that DER function through expression of Aos during lateral inhibition to mediate proper spacing of R8 cells (Spencer et al., 1998). However, exactly how Aos suppress Ato expression remained unclear.

The studies we described here indicate that M8 is a target of DER during lateral inhibition. During this process, DER cooperates with Notch to specify R8 photoreceptors, implicated by the observation that $M8S^{14}D$ (CK2+MAPK-phosphomimetic) elicits more potent eye defects, than $M8S^{4}D$ (CK2-phosphomimetic). This hyperactive behavior of $M8S^{14}D$ most likely reflects a bypass of phosphorylation by MAPK. This model is further supported by the observation that $M8S^{4}D$ can mimic the behavior of $M8S^{14}D$ in a hyperactive *egfr* background (*Elp/+*). This indicates that *Elp* may engender premature secondary phosphorylation of $M8S^{4}D$ thereby enhancing its activity. In contrast, the eye defects of $M8S^{4}D$ can be rescued by decrease dosage of DER. Collectively, these observations suggest that M8 is, indeed, a target of activated MAPK. However, the observation that the MAPK-phosphomimetic variant ($M8S^{1}D$) does not elicit any eye defect,

suggests that MAPK by itself is not sufficient, but rather complements CK2 in potentiating M8 activity. Based on these observations, we proposed that CK2 acts as 'primary gatekeeper' or 'priming' kinase, and MAPK is the 'secondary gatekeeper' or the mediator of M8 activity.

Taken together, our studies further extend the mechanisms regulating lateral inhibition. Lateral inhibition has been classically thought to be the outcome of transcription and ensuing accumulation of E(spl) proteins. Contrary to this classical view, our studies indicate that lateral inhibition is a regulated process, involving multisite/hierarchical phosphorylation of M8. Regulation by phosphorylation would be faster and more robust, compared to one that involves transcription alone. This multisite phosphorylation may function as a 'clock' that regulates the precise onset of M8 repressor activity. In the context of R8 specification, DER and Notch may impinge upon M8 to 'filter' transcriptional noise i.e., fluctuations in *ato* and *E(spl)* expression. In line with this, DER and Notch would initiate lateral inhibition only when the Ato feedback loop is established, and the threshold level for R8 specification has been achieved.

Materials and Methods:

Plasmid construction and germline transformations.

The construction of variants of M8 harboring appropriate substitutions (Ala/Asp) in place of $Ser^{151, 154, 155, 159}$ were generated by employing site-directed mutagenesis. All constructs were sequenced to confirm the presence of only the intended mutations. For *in vivo* expression, cDNA's were cloned into the EcoRI and BglII sites of the plasmid pUAST (Brand and Perrimon, 1993), and germline transformants were generated as described (Rubin, 1983). w^+ progeny were identified and the location of insertions was determined via

crosses to lines harboring chromosomes carrying dominant visible markers. At least, 6-10 independent insertions of each construct have been used in these studies.

Fly stocks, crosses and phenotypes.

Flies were raised at 24^{o}C on standard Yeast-Glucose medium. The *Gal4* drivers used in these studies were generously provided by other researchers or obtained from the Bloomington Stock Centre. These drivers are *109-68Gal4* (Jarman and Ahmed, 1998), h^{H10}*Gal4* (Huang and Fischer-Vize, 1996). The DER mutant lines *Elp* (B-1564), $egfr^{f24}$ (B-6500) were obtained from Bloomington Stock Centre.

All crosses were performed at 24^{o}C, unless otherwise mentioned. Fly heads were dehydrated by sequential passes through a graded alcohol series for 24 hours each (25-50-75-absolute). Finally, heads were passed through Hexamethyldisalizane, and mounted on EM stubs (Ted Pella). Heads were dried for 24 hours; sputter coated with gold, and examined with a JEOL-6400 scanning electron microscope at an accelerating voltage of 10-20kV. Images were acquired and processed with Adobe Photoshop and collated in Adobe Illustrator. For quantitative analysis of eye field, ≥10 flies were photographed, and ommatidial numbers were determined manually.

Immunostaining and confocal microscopy.

Imaginal discs were isolated were isolated from late third instar larvae and processed as described (Kavler et al., 1999) with modifications. Discs were fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 45 minutes at 4^{o}C, and then washed three times with PBS containing 0.1% Triton-X-100 (PBS-TX). The discs were incubated for 12 hours at 4^{o}Cin PBS-TX containing 5% normal goat serum and primary antibody. Following this, eye discs were washed three times with PBS containing 0.3% PBS-TX, immunostained for 2-3 hours in secondary antibody and mounted in Vectashield.

The following antibodies were used in this study: guinea pig anti-Sens (1:800, gift of Hugo Bellen), and mouse anti-ELAV (DSHB) at a dilution of 1:500. Secondary antibodies (Molecular Probes) were goat-anti-mouse IgG coupled to Alexa Fluor 594 (1:1000) and goat-anti-guinea pig-IgG coupled to Alexa Fluor 488 (1:1000).

Confocal Microscopy:

An Olympus FluoView (FV1000) was used for confocal imaging. Images in Fig. 2 and 3 represent scans acquired every 1μm along the apicobasal axis of the discs and the compressed as a Z stack. Individual Z-stacked images were exported as TIFF, which were processed in Adobe Photoshop and collated in Adobe Illustrator.

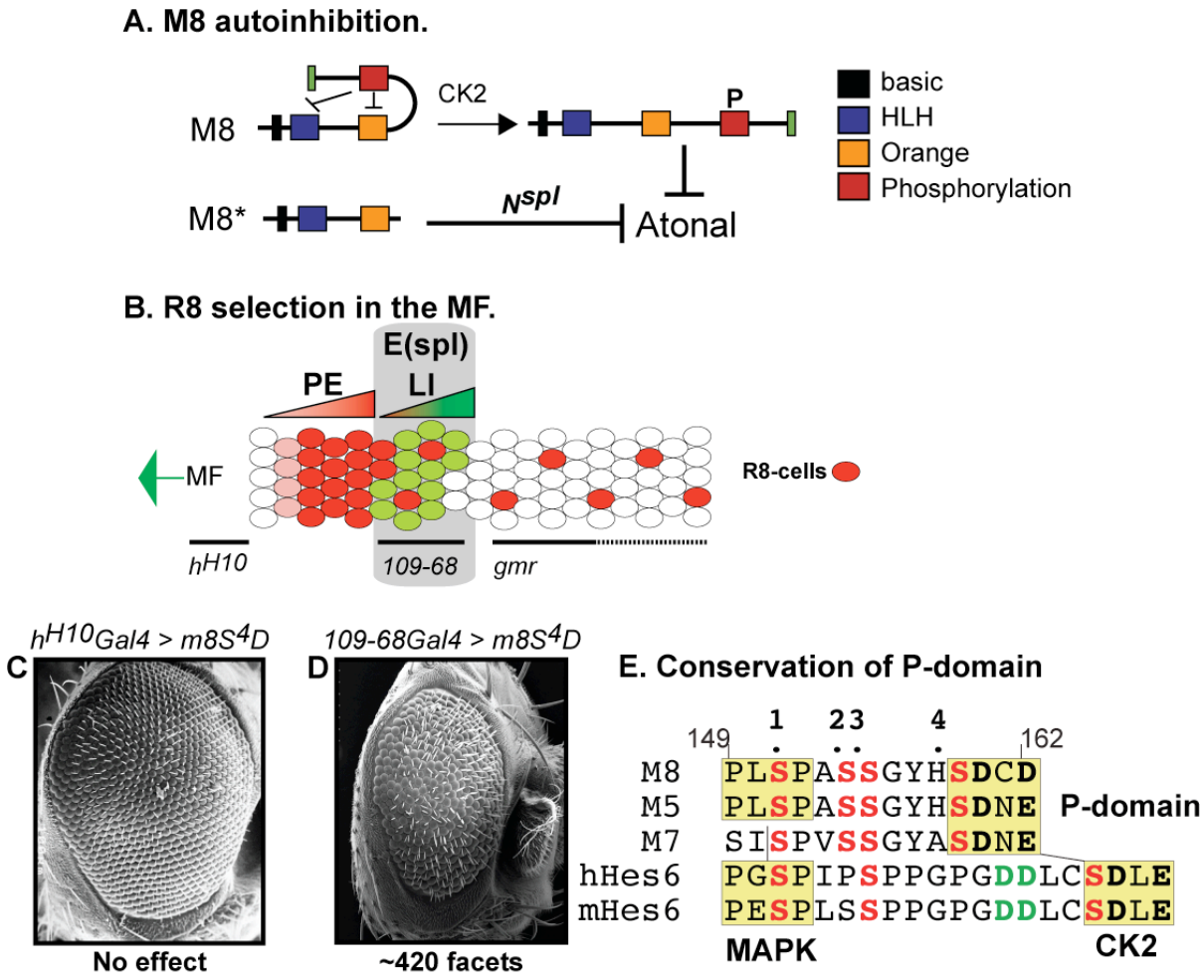

Fig. 1. Autoinhibition model and MF specificity of M8S^{4}D. (A)The CtD autoinhibits M8, and phosphorylation favors a conformational state permissive to antagonize Atonal. M8* lacks the C-terminal domain and antagonize Ato and blocks eye development in *N^{spl}* background. (B) The process of R8 selection in the MF. Schematic of cells spanning the proneural enhancement (PE), and the lateral inhibition stage of the MF are shown relative to the Gal4 drivers. *ato* expression in response to Notch signaling (PE) is denoted as red shaded box. *E(spl)* expression in response to inhibitory Notch signaling (LI) is shown as green shaded box. (C-D) Adult eye phenotypes of indicated genotypes. (E) Conservation of phosphorylation or P-domain. Conserved phosphorylation sites (black dots) in Drosophila

E(spl)M8/5/7 and human (h) and murine (m) Hes6. Numbers above alignment refer to the M8 protein. Ser^{151} and Ser^{159} meet the consensus recognition motifs for MAPK and CK2 respectively.

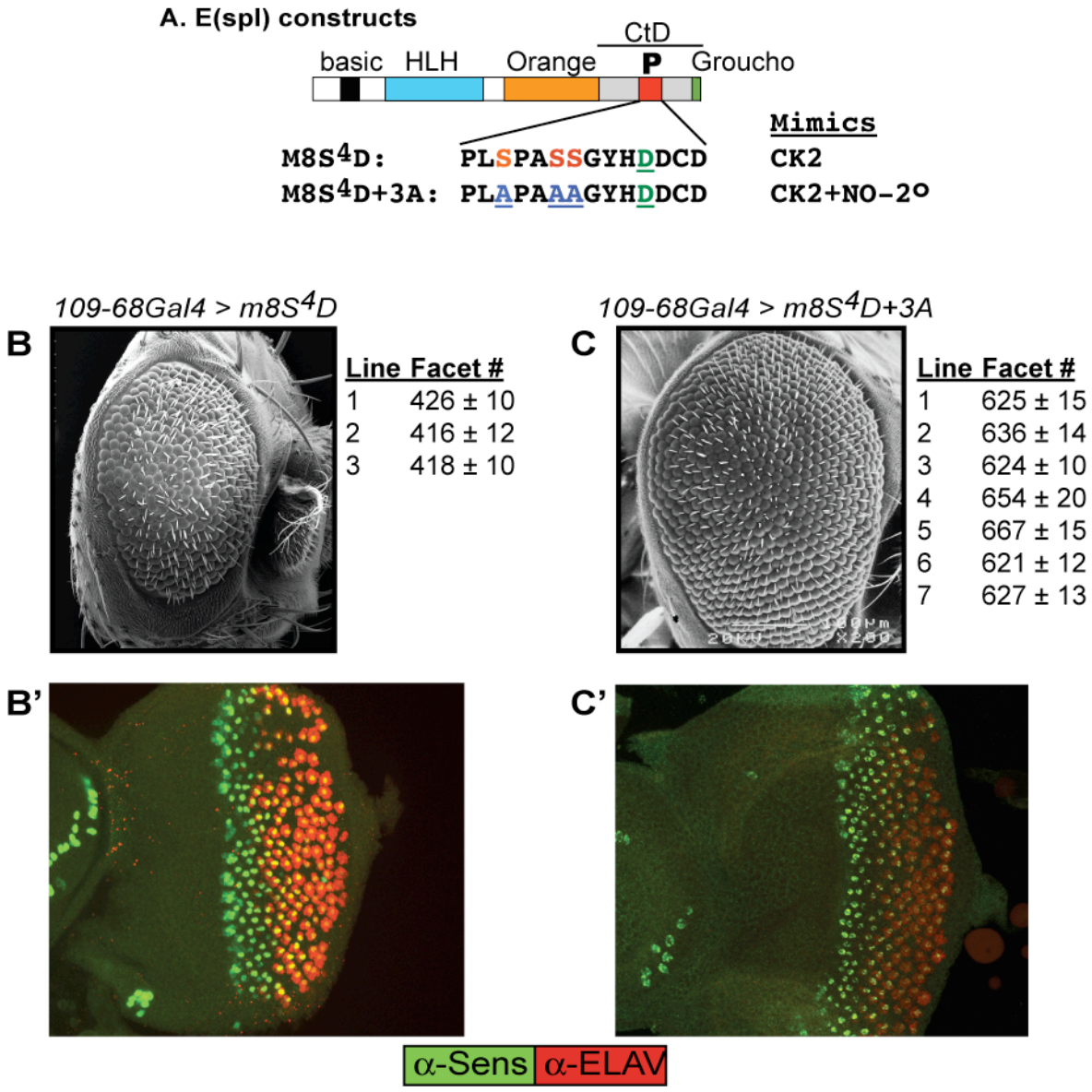

Fig. 2. The reduced eye of M8S^{4}D requires an intact P-domain. (A) Schematic of variants of M8. M8S^{4}D harbors Asp at position Ser^{159} (CK2 site). M8S^{4}D+3A harbors Asp at position Ser^{159} and Ala at the secondary phosphorylation sites (Ser$^{151, 154, 155}$). (B-C) Adult eye phenotypes. The genotypes are (B) *109-68Gal4/+; m8S^{4}D/+* and (C) *109-68Gal4/+; m8S^{4}D+3A/+*. Number of lines tested and the facet counts of the corresponding genotypes are given. Magnification is 200x. (B'-C') Eye discs were immunostained with α-Sens and α-

ELAV. Genotypes of discs in B' and C' correspond to those in panel B and C respectively, and arrows denote MF progression.

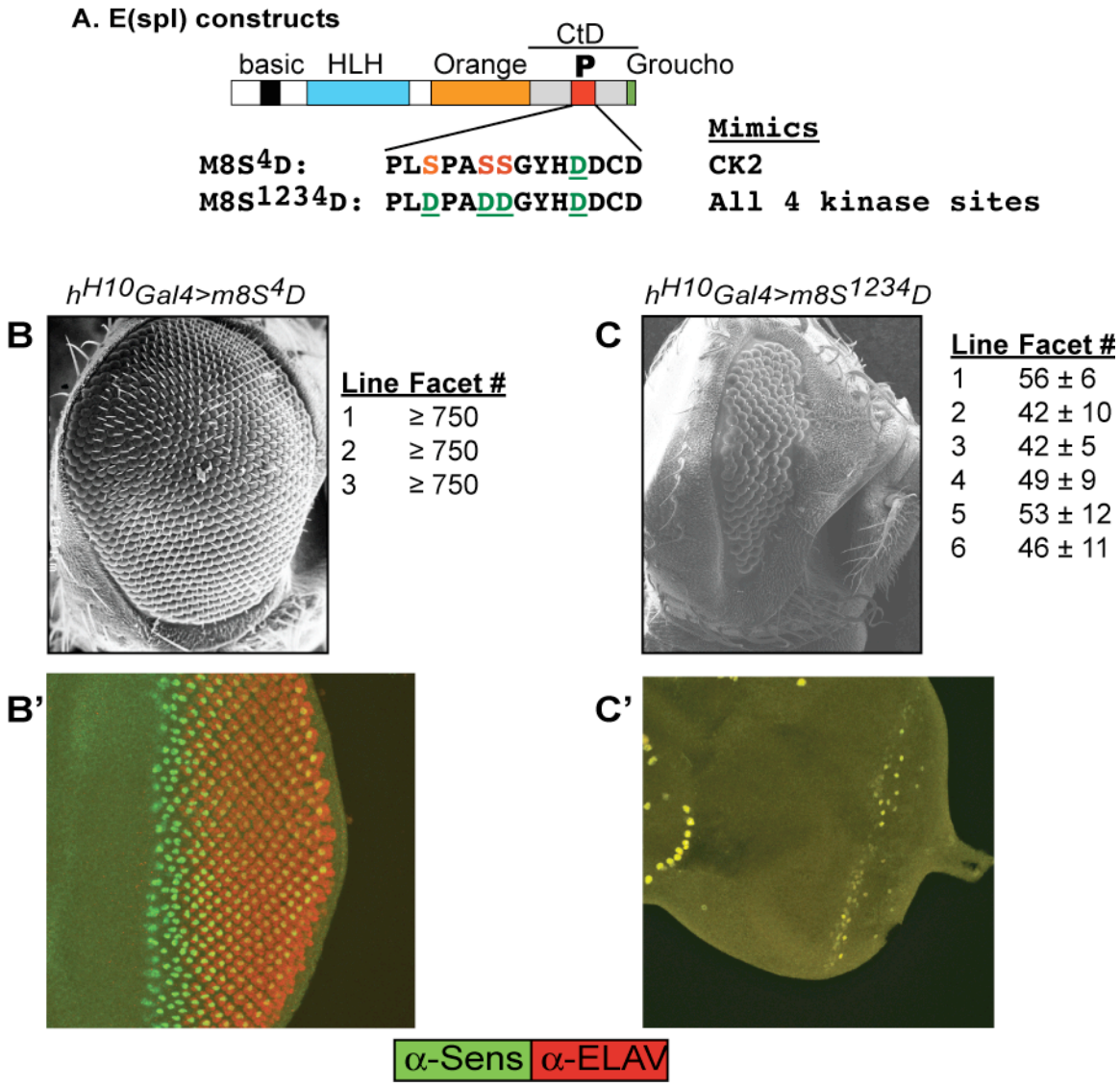

Fig. 3. Secondary phosphorylation enhances M8 activity. (A) Schematic of variants of M8. M8S^{4}D harbors Asp at position Ser^{159} (CK2 site). M8S^{1234}D harbors Asp at all the phosphorylation sites. (B-C) Adult eye phenotypes. The genotypes are (B) *m8S^{4}D/+; h^{H10}Gal4/+* and (C) *m8S^{1234}D/+; h^{H10}Gal4/+*. Number of lines tested and the facet counts of the corresponding genotypes are given. Magnification is 200x. (B'-C') Eye discs were immunostained with α-Sens and α-ELAV. Genotypes of discs in B' and C' correspond to those in panel B and C respectively, and arrows denote MF progression.

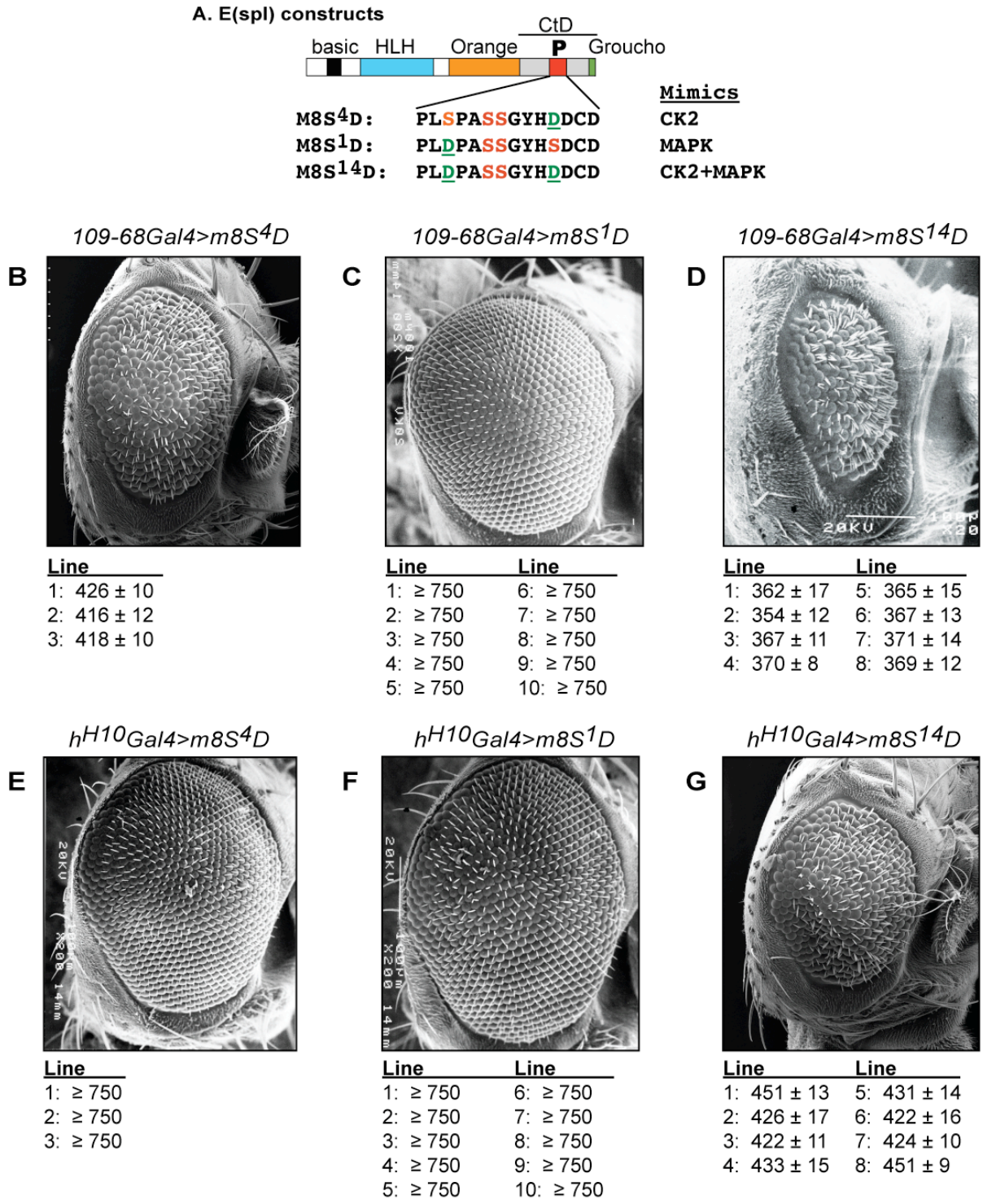

Fig. 4. The MAPK site influences M8 activity. (A) Schematic of variants of M8. M8S^{4}D harbors Asp at position Ser^{159} (CK2 site). M8S^{1}D harbors Asp at position Ser^{151} (MAPK site).

M8S^{14}D harbors Asp both at Ser^{151} and Ser^{159} (CK2+MAPK). (B-G) Adult eye phenotypes. The genotypes are (B) *109-68Gal4/+; m8S^{4}D/+,* (C) *109-68Gal4/+; m8S^{1}D/+,* (D) *109-68Gal4/+; m8S^{14}D/+,* (E) *m8S^{4}D/+; h^{H10}Gal4/+*, (F) *m8S^{1}D/+; h^{H10}Gal4/+*, (G) *m8S^{14}D/+; h^{H10}Gal4/+*. Magnification is 200x. Number of lines tested and the facet counts of the corresponding genotypes are given.

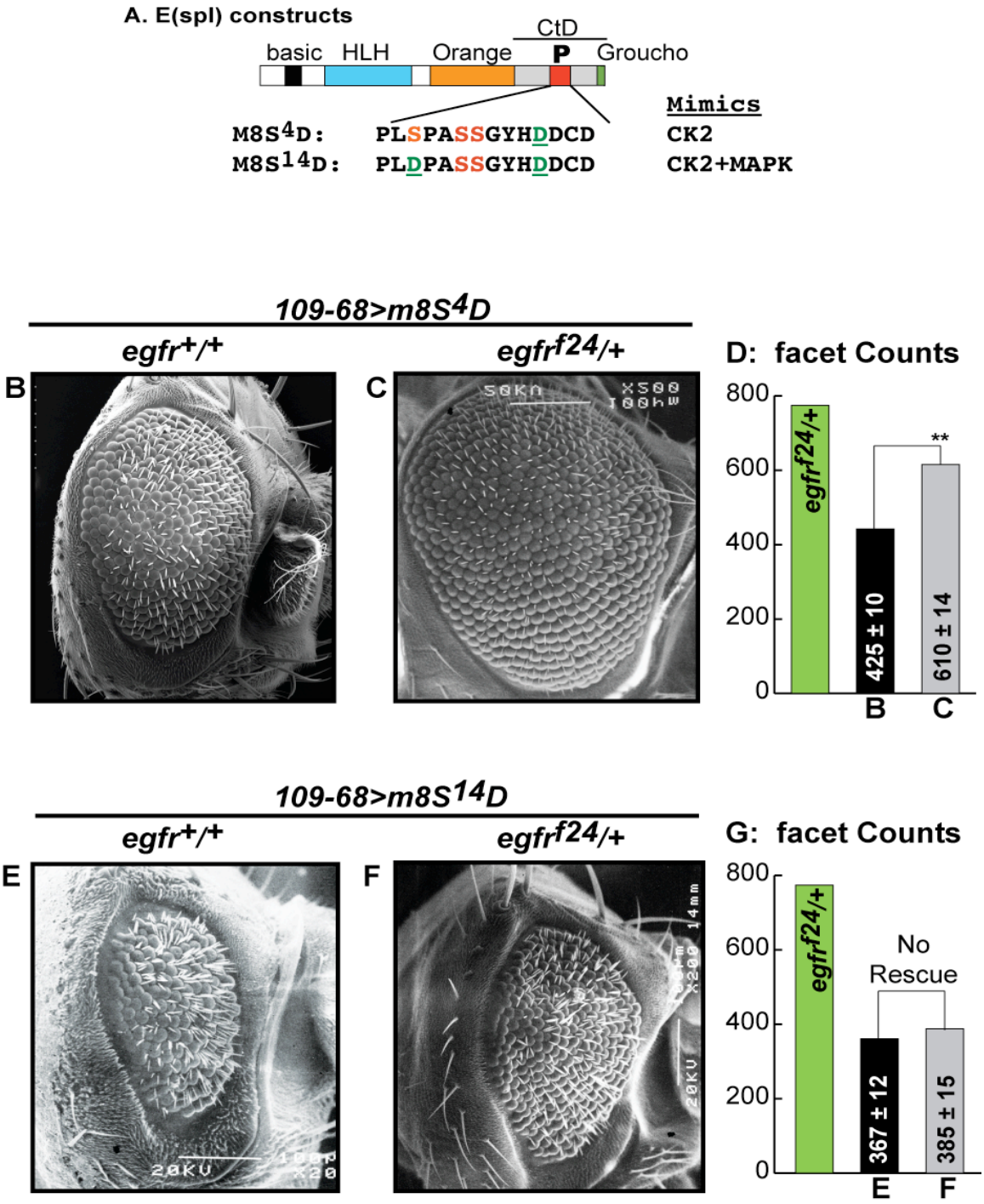

Fig. 5. The reduced eye of M8S^{4}D is rescued by decreased DER levels. (A) Schematic variants of M8. (B, C, E and F) Adult eye phenotypes. The genotypes are (B) *109-68Gal4/+;*

m8S^{4}D/+, (C) *109-68Gal4/egfr^{f24}; m8S^{4}D/+,* (E) *109-68Gal4/+; m8S^{14}D/+,* (F) *109-68Gal4/egfr^{f24}; m8S^{14}D/+*. Magnification is 200x. (D and G) Quantitative analysis of facet numbers of the indicated genotypes. Asterisk denotes p-value <0.001.

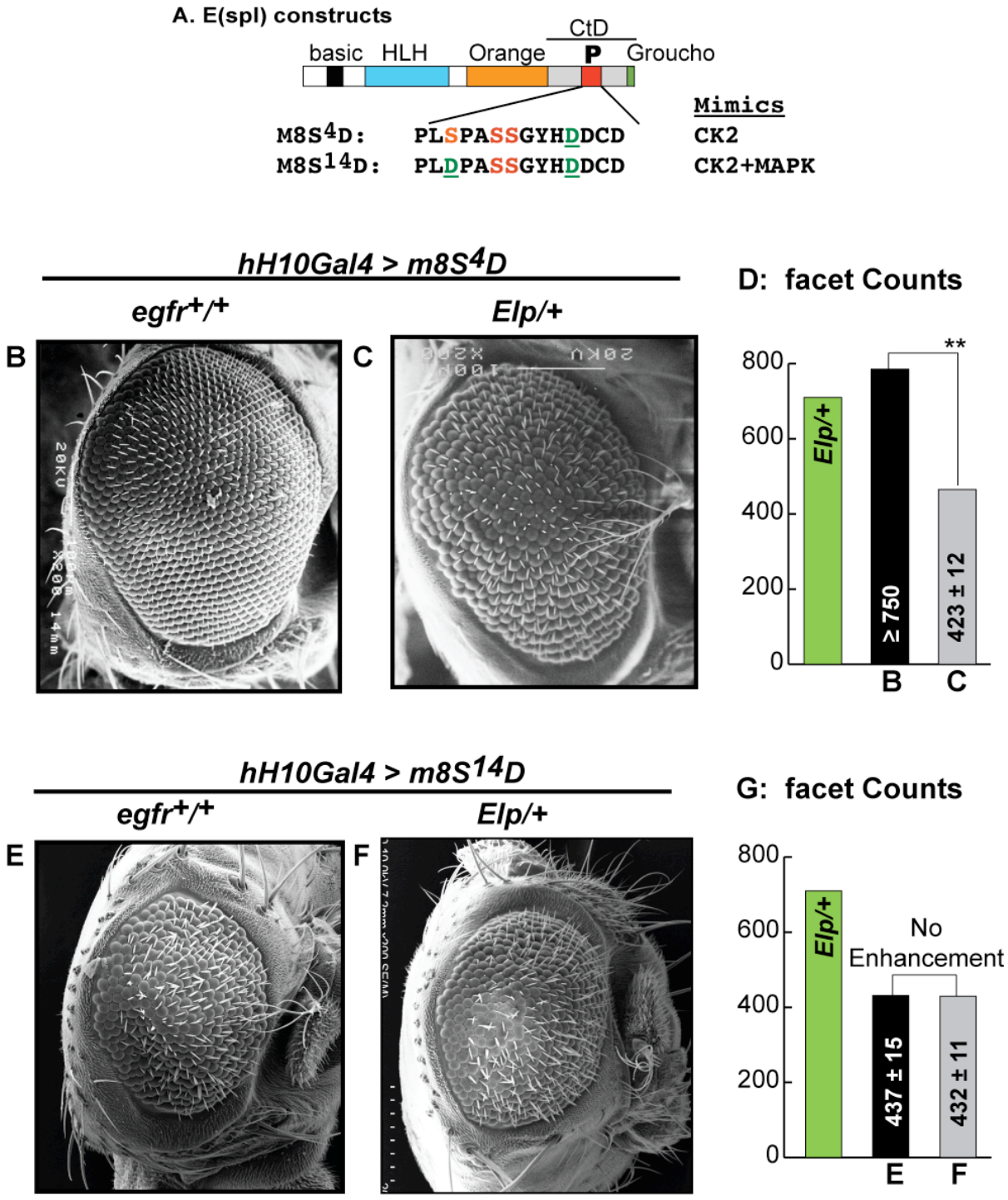

Fig. 6. Increased DER dosage enhances M8 activity. (A) Schematic variants of M8. (B, C, E and F) Adult eye phenotypes. The genotypes are (B) *m8S^{4}D /+; h^{H10}Gal4/+,* (C)

m8S^{4}D/Elp; h^{H10}Gal4/+, (E) *m8S^{14}D /+; h^{H10}Gal4/+,* (F) *m8S^{14}D /Elp; h^{H10}Gal4/+*. Magnification is 200x. (D and G) Quantitative analysis of facet numbers of the indicated genotypes. Asterisk denotes p-value <0.001.

Chapter 5

Genetic interactions between *CK2*, *Notch,* and *E(spl)* during Drosophila neurogenesis.

Abstract:

In recent years, a number of reports have indicated that protein kinase CK2 potentiates neural repression by E(spl) proteins. This role has emerged from reverse genetic analyses of CK2-site specific variants of M8, or by targeted reduction of CK2 levels and/or activity. However, direct genetic evidence for a role for CK2 in inhibitory Notch signaling has hitherto been lacking. Here, we report direct genetic interactions between alleles of *CK2, m8,* and *Notch*. *Timekeeper (Tik)*, a dominant negative allele of CK2, interacts with the *m8* allele, *E(spl)D,* and elicits ectopic, split, and missing bristles, all of which are hallmarks of impaired Notch signaling. In addition, *Tik* interacts with hypomorphic *Notch* alleles, and elicits bristle and 'notched' wing defects that closely mimic loss of Notch functions. Furthermore, we find that *Tik* rescues the severe reduced eye of N^{spl}/Y; *E(spl)D/+* flies, a genetic interaction that led to the original identification of the *E(spl)C*. Specifically, *Tik* restores the R8 photoreceptors, whose specification is blocked by the *E(spl)D* allele. Rescue by *Tik* is likely to reflect effects mediated via the $E(spl)^{+}$ allele. Together, these studies provide direct genetic evidence for CK2 as a mediator of Notch signaling.

Introduction:

Development of sensory organs such as the eye or bristles in Drosophila is the outcome of lateral inhibition, which is mediated by Notch signaling (reviewed in Artavanis-Tsakonas et al., 1999; Baonza and Freeman, 2001; Bray, 1997; Mumm and Kopan, 2000). Neural cell-fate specification initiates with the expression of the proneural transcription factors, *atonal* (*ato*) or *achaete-scute complex* (*ASC*) that bestow on cells the ability to become neural precursors (Campos-Ortega, 1997; Dambly-Chaudiere and Vervoort, 1998; Massari and Murre, 2000). These transcription factors drive the formation of a group of equipotent cells termed the proneural cluster (PNC's). From each PNC, however, only one cell adopts the neural cell fate. This cell is termed the R8 photoreceptor in the eye, and the sensory organ precursor (SOP) in the bristle. The selection of the R8 or the SOP is dictated by inhibitory Notch signaling and is mediated through the E(spl) proteins, which antagonize Ato/ASC (Artavanis-Tsakonas et al., 1995).

Accumulating evidence indicates that phosphorylation of the E(spl) member M8 by protein kinase CK2 potentiates repressor activity during lateral inhibition. This regulation was initially proposed to be necessary for R8 selection (Karandikar et al., 2004). Follow-up studies have implicated a role for CK2 in SOP selection as well (Bose et al., 2006). However, direct genetic interactions, generally considered to be a benchmark, have hitherto been lacking. The studies described here demonstrate just such interactions between alleles of *CK2*, *E(spl)*, and *Notch*. The developmental defects provide strong evidence that CK2 is present and operational in cells undergoing lateral inhibition, and indicate that repression by E(spl) proteins does involve posttranslational modification (phosphorylation).

Results:

CK2α* interacts with the *m8* allele *E(spl)D.

We have previously observed that the *CK2* allele *Timekeeper* (*Tik*) interacts with hypomorphic *hairy (h)* alleles and elicits developmental defects that are akin to *h* loss of function (Kahali et al., 2008). These studies suggested that *Tik* might provide a 'sensitized' background to study the role of CK2 during inhibitory Notch signaling, a process that has generally been thought to be independent of posttranslational regulation via phosphorylation.

We employed two alleles of *CK2α*, i.e., *Tik* and $CK2^{MB00477}$ (Fig. 1A). *Tik* is a dominant-negative allele of *CK2α,* which lacks kinase activity (Lin et al., 2002). In the heterozygous state, *Tik* elicits severe clock defects, and is lethal when homozygous (Lin et al., 2002). Moreover, lethality manifests at the first larval instar. Since eye and bristle development do not initiate until the third larval instar, the analysis of *Tik* in these contexts has not been possible. In addition, mitotic clones are cell autonomous lethal (Andreas Jenny, personal communication), precluding studies on the role of CK2. Surprisingly, *Tik/+* animals do not display overt neural (eye or bristle) defects (Bose et al., 2006) indicating that CK2 activity in this background is still sufficient for Notch signaling. The second allele, $CK2^{MB00477}$, is a hypomorph that harbors a *minos* insertion in the 5' UTR (Bellen et al., 2004). $CK2^{MB00477}$ is pupal lethal when homozygous, or in combination with *Tik* (Bose and Bidwai, unpublished), and analogous to *Tik/+* animals does not perturb eye or bristle patterning when heterozygous.

We first tested for interactions between *E(spl)D* and alleles of *CK2α.* On their own, ~12% of *E(spl)D/+* animals display bristle defects (Fig. 2C), which includes missing but not ectopic bristles (Fig. 2D, E). In contrast, *Tik/+* or $CK2^{MB00477}$*/+* animals do not display any discernible missing bristles; the only observable defect is a low level (~5%) of ectopic bristles (Fig. 2C, D, E). In the transheterozygous state, however, ~85-90% of *Tik/E(spl)D*

animals display both ectopic and missing bristles (Fig. 2A, B, C, D, E). Consistent with the possibility that it is a hypomorphic allele, ~40% of *CK2*^{MB00477}*/E(spl)D* animals display ectopic and missing bristles (Fig. 2C, D, E). This is the first demonstration of a direct interaction between *CK2* and *E(spl)*, and indicate that *CK2*^{MB00477} is a hypomorph. The elicitation of similar bristle defects would indicate that a second site mutation unrelated to *Tik* or *CK2*^{MB00477} is unlikely to underlie their interactions with *E(spl)D*, because these two *CK2* alleles have been identified independently in two different laboratories over a period of eight years.

E(spl)D is the only known allele of any *E(spl)* member. This allele encodes for a truncated M8 protein that lacks the phosphorylation site for CK2 (Fig. 1B). Consequently, M8 dosage in an *E(spl)D/+* background is halved. If M8 phosphorylation is necessary for repression, the genetic interactions between *Tik* and *E(spl)D* may reflect hypophosphorylation of wild type M8, one encoded by the *E(spl)*$^{+}$ allele. We therefore tested if *Tik* or *CK2*^{MB00477} genetically interact with deficiencies that uncover the *E(spl)C.* We employed two deficiencies; these are *Df(3R)*^{BX22} and *DF(3R)*^{Boss14} (Fig. 1B), which in the heterozygous state display no discernible bristle abnormalities, ectopic or missing (Fig. 2F, G). Surprisingly, neither *Tik* nor *CK2*^{MB00477} displayed an interaction with either deficiency (Fig.2F, G). While these results may seem counterintuitive, it should be noted that the *E(spl)D* encoded protein (M8*) has antimorphic activity, which is not the case with either deficiency (see discussion).

***CK2* interacts with hypomorphic *Notch* alleles.**

The ectopic and missing bristle defects of *Tik/E(spl)D* or *CK2*^{MB00477}*/E(spl)D* transheterozygous animals are reminiscent of phenotypes associated with impaired Notch signaling. Therefore, to further evaluate a role for CK2 in Notch signaling, we tested for interactions between alleles of *CK2* and *Notch*.

For the studies, we employed two *Notch* alleles, N^{264-39} and N^{1}. The former is an amorphic allele, which exhibits 'notched' wings and bristles abnormalities in the heterozygous state (Fig. 3D). In contrast, N^{1} is a hypomorphic allele that exhibits 'notched' wing phenotype whose penetrance is somewhat muted, but does not perturb bristle patterning (Fig. 3E). Both of these *Notch* alleles are embryonic lethal when homozygous or hemizygous.

Neither *Tik* nor $CK2^{MB00477}$ exhibit any wing defects. Importantly, animals transheterozygous for *CK2* and *Notch* alleles exhibit 'notched' wings with a penetrance that is higher than the individual mutations. Once again, the penetrance of wing defects is higher with *Tik*, supporting the hypomorphic nature of $CK2^{MB00477}$. In addition, N^{264-39}/+; *Tik/+* animals display a greater penetrance of ectopic, split and missing bristles, all of which are hallmarks of Notch loss of function. No enhancement of N^{1} by *Tik* was evidenced in the bristle, consistent with the weak hypomorphic nature of this *Notch* allele. Nevertheless, the wing and bristle defects demonstrate that *CK2* genetically interacts with *Notch*.

Tik rescues the reduced eye defects of N^{spl}/Y; E(spl)D.

The severely reduced eye of N^{spl}/Y; E(spl)D/+ (Fig. 4A) has been instrumental in providing important mechanistic insights into Notch mediated lateral inhibition (Giebel and Campos-Ortega, 1997; Kahali et al., 2009; Kahali et al., 2010; Karandikar et al., 2004; Nagel et al., 1999; Nagel and Preiss, 1999). It has been previously shown that the retinal defects of N^{spl}/Y; E(spl)D/+ are partially rescued by reduced *E(spl)* dosage, raising the possibility that the wild type $E(spl)^{+}$ allele contributes to the eye defects (Shepard et al., 1989). Since both *Tik* and $CK2^{MB00477}$ elicit a Notch loss of function phenotype in combination with *E(spl)D* or *Notch* alleles, we next tested whether these *CK2* alleles can rescue the reduced eye of N^{spl}/Y; E(spl)D/+.

We find that *Tik*, indeed, rescues the reduced eye of N^{spl}/Y; *E(spl)D/+* animals (Fig. 4B). In contrast, no such rescue was observed with $CK2^{MB00477}$ (Fig. 4C). To quantify the extent of rescue, we determine facet counts, which were determined for ~10 flies of all relevant genotypes. In this analysis, the facet numbers of N^{spl}/Y; *E(spl)D/+* (10±5) was considered as the baseline (Fig. 4D). Consistent with the adult eye, *Tik* significantly increases the facet numbers (140±12) of N^{spl}/Y; *E(spl)D/+* animals, whereas no restoration was observed in N^{spl}/Y; *E(spl)D/*$CK2^{MB00477}$ animals, whose facet numbers were indistinguishable from the baseline (Fig. 4D). Neither *CK2* allele modified the rough and reduced eye of N^{spl} males (Fig. 4D), indicating that rescue of N^{spl}/Y; *E(spl)D/+* animals by *Tik* is likely to involve effects mediated through the $E(spl)^{+}$ allele.

***Tik* restores R8 specification:**

The reduced eye of N^{spl}/Y; *E(spl)D/+* reflects loss of founding R8 photoreceptors whose specification is essential for later stages of retinal histogenesis. We have previously demonstrated that the expression of an antimorphic variant M8 rescues the eye and R8 defects of N^{spl}/Y; *E(spl)D/+* animals (Kahali et al., 2009). We therefore stained third instar larval eye discs for Senseless (Sens) expression, which marks differentiated R8s (Frankfort et al., 2001; Nolo et al., 2000). In the wild-type eye discs (not shown), Sens levels are maintained throughout the DV and AP axis of the eye field relative to the morphogenetic furrow. In the case of N^{spl}/Y; *E(spl)D* animals, there is a significant loss of Sens+ cells (Fig. 4A'), and this loss is mitigated by *Tik* (Fig. 4B'). Therefore, rescue of the reduced eye by *Tik* reflects restored specification of the R8 cells.

In summary, the multiple lines of evidence provide strong support for a role for CK2 in Notch signaling, and indicate that the E(spl) repressor M8 is an in vivo target for phosphorylation by this protein kinase. The neural defects that we report would be consistent with phosphorylation potentiating repression by E(spl).

Discussion:

Notch signaling is the driver of lateral inhibition, during which the expression of E(spl) repressors mediates the selection of a single R8 or SOP from clusters of equipotential cells. This selection requires the antagonism of the proneural activators Ato or ASC, a process that requires direct interactions between these two groups of bHLH proteins (Artavanis-Tsakonas et al., 1999; Baonza et al., 2001; Mumm and Kopan, 2000). Moreover, it has been thought that this antagonism predominantly reflects the dosage of the E(spl) proteins, a view that has emerged from generalized loss of thoracic bristles when these repressors are ectopically expressed in the corresponding PNC's. However, follow-up studies indicate that antagonism is a regulated process, one that involves phosphorylation by protein kinase CK2. In the case of M8, for which most evidence is available, this modification appears to convert M8 from an autoinhibited state to one that is competent for binding to Ato and repression of the R8 fate. Such a role for CK2 has also been evidenced in the SOP lineage, based on the findings that targeted reduction of CK2 levels/activity elicits ectopic and split bristles, which implicate phosphorylation during N-dependent SOP selection and the socket-to-shaft dichotomy, respectively (Fig. 5). However, the absence of direct gene interactions, a benchmark, has weakened arguments for a role for CK2. The studies we describe here provide multiple lines of direct evidence that CK2 indeed regulates the outcome of Notch signaling.

The exacerbated bristle defects of *E(spl)D/Tik* or *E(spl)D/CK2*^{MB00477} animals (Fig. 2) are hallmarks of impaired lateral inhibition. For example, the extra bristles of *E(spl)D/Tik* or *E(spl)D/CK2*^{MB00477} animals are likely to reflect loss of E(spl) activity during SOP selection, and these extra SOPs would developed into ectopic closely spaced bristles. In contrast, the high penetrance of missing bristles in *E(spl)D/Tik* or *E(spl)D/CK2*^{MB00477} animals (Fig. 2) could reflect loss of E(spl) activity after the completion of SOP selection (see Fig. 5). In this

case, the asymmetric division of the pIIa and pIIb neuroblasts, which requires Notch and E(spl), could be rendered defective upon loss of CK2 functions. Consequently, the pIIa cell would adopt the pIIb fate, thereby leading to the loss of the (external) socket and sheath cells. In addition, loss of CK2 could also lead to impaired division of the pIIIb neuroblast, thereby leading to the specification of excess neurons, at the expense of the sheath cell. These observations suggest that the bristle defects of reduced *CK2* dosage in an *E(spl)D/+* background may reflect reduced (hypo) phosphorylation of E(spl) members.

Although, *E(spl)D* exhibits interactions with *CK2*α alleles, this was not the case with deficiencies that uncover the *E(spl)C* (Fig. 2). At face value, the latter results would suggest that halved dosage of *E(spl)* members in conjunction with reduced CK2 activity is still sufficient for lateral inhibition to occur properly. If so, why do CK2 alleles only interact genetically with *E(spl)D*? We think that the unique nature of the *E(spl)D* mutation is the principle reason. Although this allele behaves in a dominant manner in the eye (see below), analysis of its encoded protein indicates that it behaves as an antimorph in the SOP lineage (Kahali et al., 2009; Nagel et al., 1999; Tietze et al., 1992). This allele encodes a truncated protein called M8* that lacks the 56 C-terminal residues, including the Gro recruitment and the CK2 phosphorylation motifs (Fig. 1B), but is fully efficient at dimerization with full length M8 (Kahali & Bidwai unpublished). Given that repression by E(spl) requires dimerization, the possibility arises that M8*-M8 dimers are impaired for lateral inhibition. Consistent with this, the ectopic expression of a *UAS-m8** construct in the PNCs (with the enhancer traps *scaGal4*, *109-68Gal4* or *G455.2* that give rise to the notal bristles), leads to ectopic bristles with a penetrance that is much higher than that in *E(spl)D/+* animals. We therefore suggest that *Tik* or *CK2*^{MB00477} accentuate the weak hypomorphic activity of *E(spl)D*. In this case, hypophosphorylation of full length M8 (encoded by the *E(spl)+* allele) would favor the inactive state, thereby exacerbating the baseline bristle defects of *E(spl)D*. In contrast, the

reduced dosage of *E(spl)* rendered by the two *E(spl)* deficiencies would be still sufficient, and a reduction of CK2 dosage would be expected to have muted/absent effects. Consistent with this suggestion, neither deficiency perturbs bristle patterning on its own.

The second line of evidence for a role for CK2 in Notch signaling is direct genetic interactions between the corresponding alleles, leading to wing margin and bristle defects akin to Notch loss of function. Analysis of a complete loss of CK2 activity has been precluded, because both alleles are homozygous lethal, and mitotic clones are cell autonomous lethal (Andreas Jenny, personal communication).

The rescue of the severely reduced eye of N^{spl}*/Y; E(spl)D/+* by *Tik* represents a third line of evidence that *CK2* is a participant in Notch pathway. N^{spl} is a gain of function allele that renders R8 precursors hypersensitive to inhibitory Notch signaling. The few R8's that are formed fail to maintain Sens expression. This, in turn, impairs the R8-dependent expression of Hedgehog (Hh) and Decapentapelagic (Dpp), whose activities are critical for *ato* expression (Li et al., 2003). In this sensitized background, *E(spl)D* further decreases Ato levels by affecting the Ato 'positive feedback' loop, leading to an almost complete loss of R8s, resulting in the ablation of the eye field (Kahali et al., 2009). Importantly, the eye defects of *E(spl)D* are rescued by reducing the dosage or activity of *E(spl)+* (Shepard et al., 1989). Given the lack of any interaction between CK2 alleles and N^{spl} *per se*, it would thus appear to be the case that rescue by *Tik* (Fig. 4) reflects, once again, the hypophosphorylation of wild type M8. The significant restoration of Sens expressing cells that we find (Fig. 4) are consistent with this suggestion, as reduced *E(spl)* activity would favor R8 specification and differentiation. Thus the rescue by *Tik* reflects restored R8 patterning

In summary, the genetic interactions and multiple developmental contexts demonstrate that CK2 is a participant of Notch signaling. While a large number of studies

have focused on the diverse mechanisms that control biosynthesis of this receptor, its processing in the ER and Golgi, the mechanisms that lead to its activation, and its downstream targets, direct genetic evidence for regulation by phosphorylation remained to be shown. Our findings would be consistent with the proposal that protein kinase CK2 regulates Notch signaling through modification of the effectors of this pathway, the E(spl) repressors.

Materials and Methods:

Fly stocks, crosses and phenotypes.

Flies were raised at 24°C on standard Yeast-Glucose medium. The mutant lines N^{1} (B6873), $N^{264\text{-}39}$ (B730), *E(spl)D* (B2447), N^{spl} (B118, B182), $CK2^{MB00477}$ (B22812) were obtained from the Bloomington Stock Centre. Dr. Ravi Allada generously provided the *Tik* and *TikR* stocks.

All crosses were performed at 24°C. Fly heads were dehydrated by sequential passes through a graded alcohol series for 24 hours each (25-50-75-absolute). Finally, heads were passed through Hexamethyldisalizane, and mounted on EM stubs (Ted Pella). Heads were dried for 24 hours; sputter coated with gold, and examined with a JEOL-6400 scanning electron microscope at an accelerating voltage of 10-20kV. Images were acquired and processed with Adobe Photoshop and collated in Adobe Illustrator. For bristle phenotypes, newly eclosed adults were photographed with Leica MZ16 stereomicroscope. For quantitative analysis of the bristle phenotypes, multiple crosses were established (≥triplicates), and adults were scored. For quantitative analyses of the eye field, ≥15 adults were photographed, and ommatidial numbers were determined manually.

Immunostaining and confocal microscopy.

Imaginal discs were isolated from late third instar larvae and processed as described with modifications. Discs were fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 45 minutes at 4^{o}C, and washed three times with PBS containing 0.1% Triton X-100 (PBS-TX). The discs were incubated for 12 hours at 4^{o}C in PBS-TX containing 5% normal goat serum and primary antibody, washed three times with PBS containing 0.3% Triton X-100 (PBS-TX) and immunostained for 2-3 hours in secondary antibody. Guinea pig anti-Sens (gift of Hugo Bellen) was used at a dilution of 1:500. Secondary antibody (Molecular Probes) was goat-anti guinea pig-IgG coupled to Alexa Fluor 488 (1:1000). Discs were mounted in vectashield. An Olympus FluoView (FV1000) was used for confocal imaging. Image in Fig. 4 represent scans acquired every 1μm along the apicobasal axis of the discs and the compressed as a Z stack. Individual Z-stacked images were exported as TIFF, which were processed in Adobe Photoshop and collated in Adobe Illustrator.

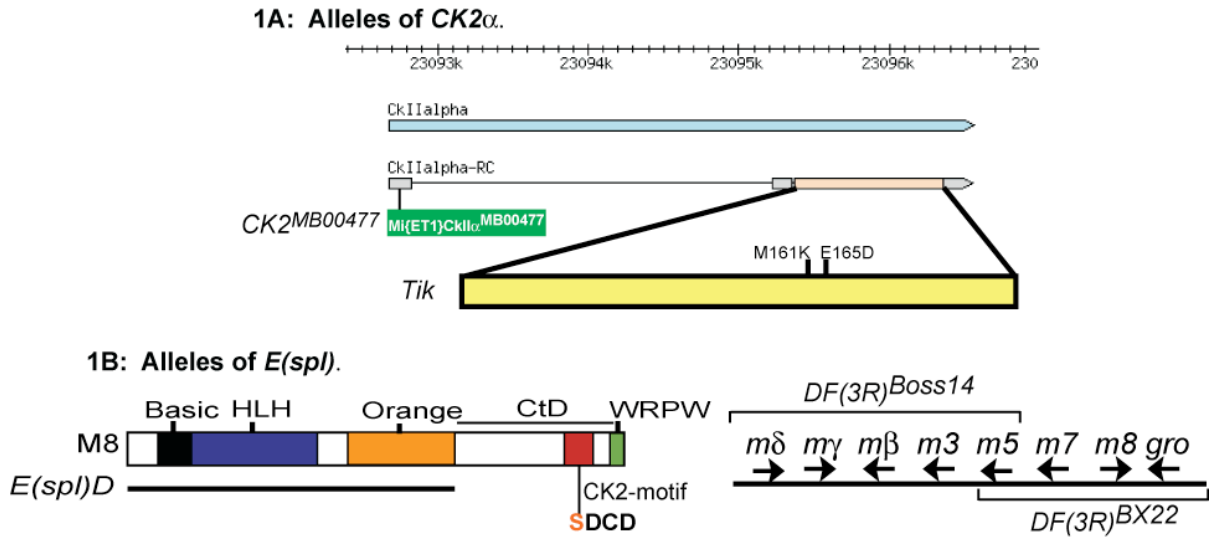

Fig. 1. Alleles of *CK2α* and *E(spl)*. (A) The alleles of Drosophila *CK2α, CK2*^{MB00477} and *Tik* and their specific lesions are indicated. (B) Alleles of *E(spl), E(spl)D, Df(3R)*^{BX22} and *Df(3R)*^{Boss14}. *E(spl)D* encodes a truncated protein, M8* that lacks the 56 C-terminal residue including the CK2 motif. *Df(3R)*^{BX22} uncovers *m5, m7,m8* and *gro*. In contrast, *Df(3R)*^{Boss14} uncovers *mδ, mγ, mβ, m3* and *m5*.

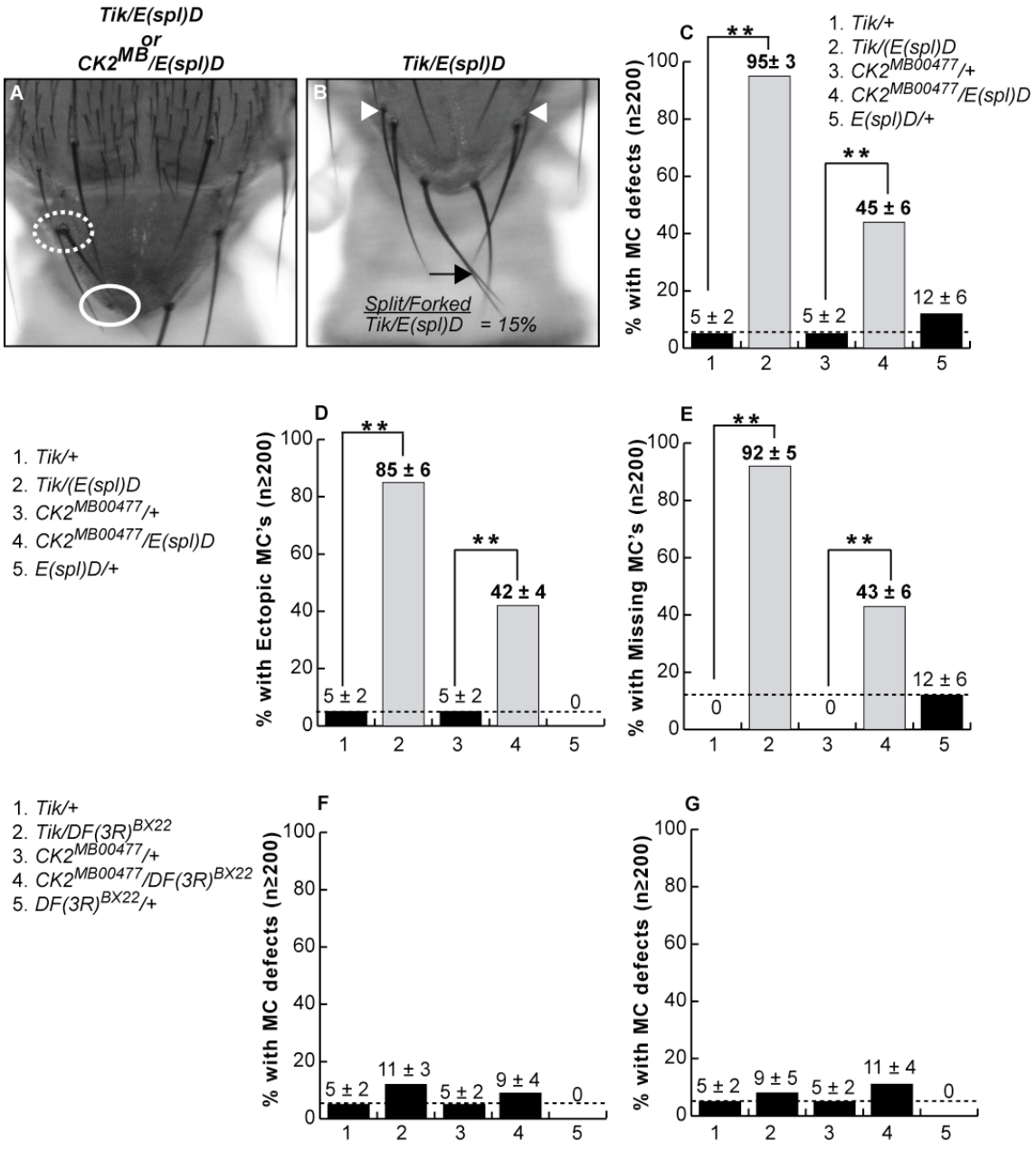

Fig. 2. Genetic interactions between *CK2α* and *m8*. (A-B) Bristle phenotypes; genotypes are as indicated. Solid circle denotes missing MC's, dotted circle denotes split MC's, arrowheads denote ectopic MC's and arrow denotes forked MC's. (C-E) *CK2α* alleles exacerbate the MC defects (ectopic, missing and split) of *E(spl)D* (grey bars). (F-G)

CK2α alleles displayed no interaction with either of the *E(spl)* deficiency. Asterisk denotes p-value <0.001.

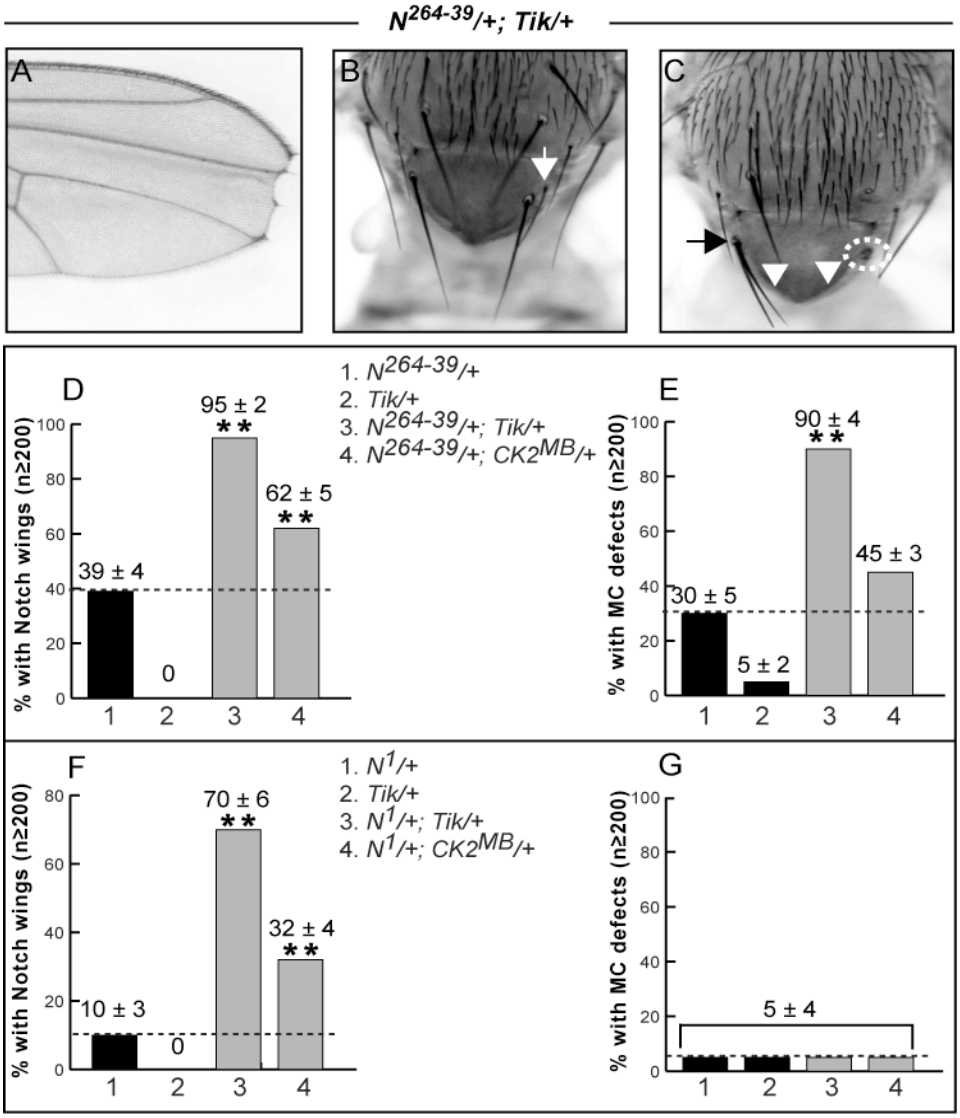

Fig. 3. Genetic interactions between *CK2α* and *Notch.* (A) Notched wing defects of N^{264-39}/+; *Tik*/+ flies. (B-C) Bristle phenotypes elicited by N^{264-39}/+; *Tik*/+ flies. White arrow denotes ectopic MC's, black arrow denotes split MC's, arrowheads denote missing MC's and dotted circle denotes socket cells. (D-E) *CK2α* alleles exacerbate the inherent MC and 'notch' wing defects of *Notch* allele, N^{264-39}. (F-G) Modulations of MC and 'notch' wing defects of *Notch* allele, N^{1} by alleles of *CK2α*. Asterisk denotes p-value <0.001.

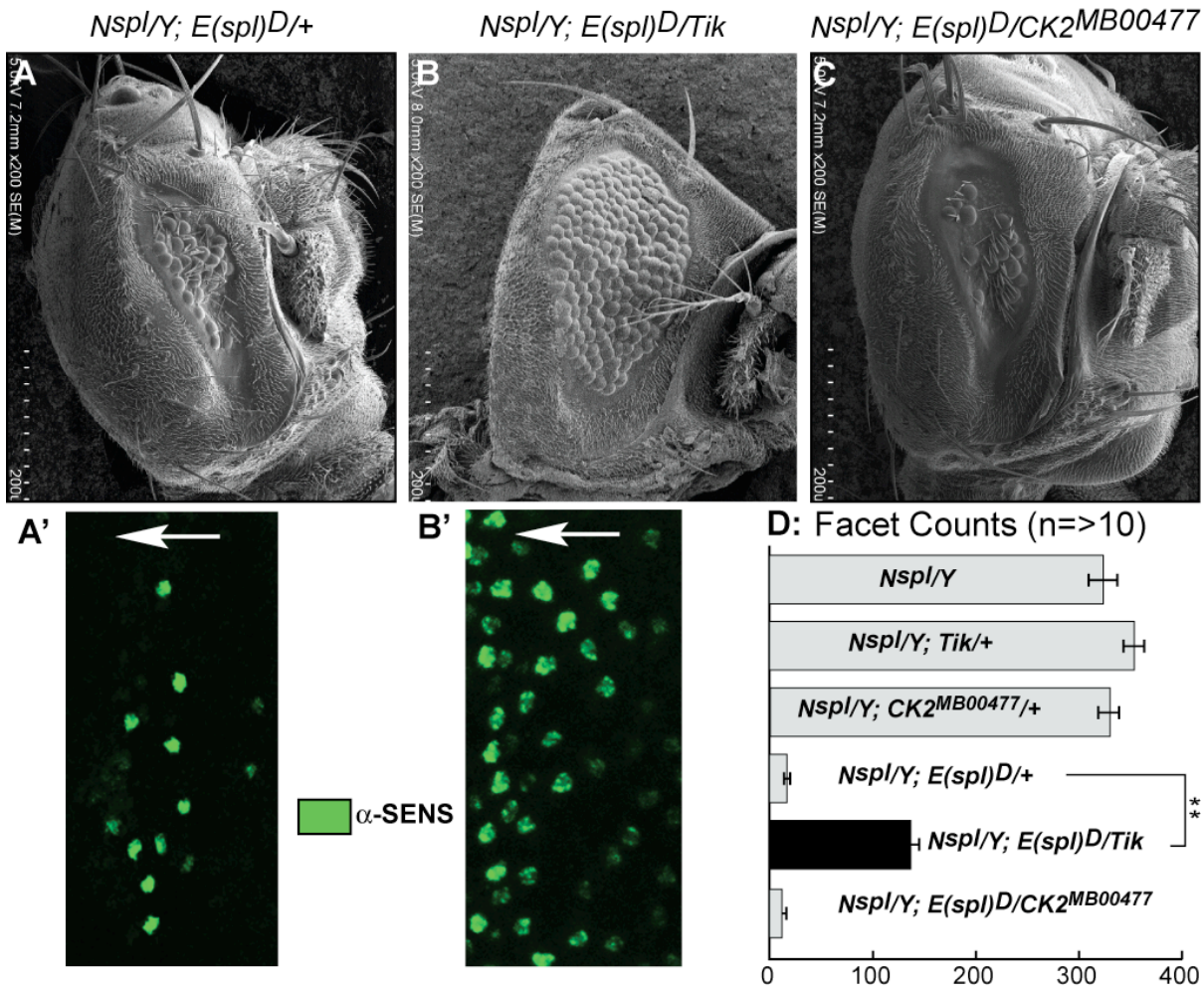

Fig. 4. *Tik* rescues the severely reduced eye of N^{spl}/Y; E(spl)D/+ flies. (A-C) Adult eye phenotypes; genotypes are as indicated. Magnification is 200x. (A' and B') Eye discs were immunostained with α-Sens. Genotypes of discs in A' and B' correspond to those in panel A and B respectively, and arrows denote MF progression. (D) Quantitative analysis of facet numbers. Facet counts were determined for ≥10 individuals for each genotype. Asterisk denotes p-value <0.001.

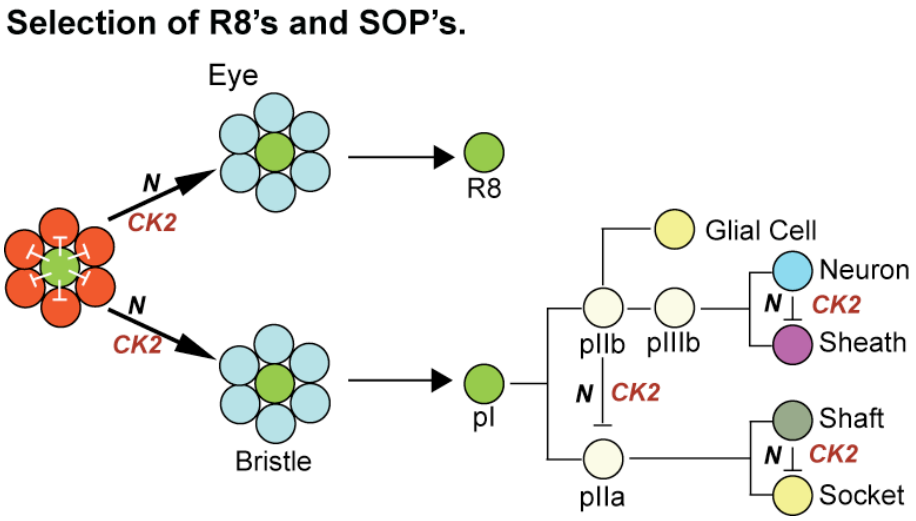

Fig. 5. Selection of R8's or SOP's from proneural clusters SOP lineages in bristle. The cell expressing the highest amount of Atonal/ASC, elicits inhibitory Notch signaling (white line). The bristle SOP gives rise to the pI neuroblast that divides asymmetrically to generate the pIIa and pIIb cells. pIIa divides to form the shaft and socket cells. pIIb gives rise to pIIIb and glial cell. Division of rhe pIIIb cell gives rise to the neuron and sheath cells. Each of these division requires inhibitory Notch signaling.

Chapter 6

Drosophila CK2 phosphorylates Hairy and regulates its activity in vivo.

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Abstract:

Hairy is a repressor that regulates bristle patterning, and its loss elicits ectopic bristles (neural hyperplasia). However, it has remained unknown whether Hairy is regulated by phosphorylation. We describe here the interaction of protein kinase CK2 and Hairy. Hairy is robustly phosphorylated by the CK2-holoenzyme (CK2-HoloE) purified from Drosophila embryos, but weakly by the catalytic CK2α subunit alone, suggesting that this interaction requires the regulatory CK2β subunit. Consistent with this, Hairy preferentially forms a direct complex with CK2-HoloE. Importantly, we demonstrate genetic interactions between CK2 and *hairy* (*h*). Thus, flies trans-heterozygous for alleles of *CK2*α and *h* display neural hyperplasia akin to homozygous hypomorphic *h* alleles. In addition, we show that similar phenotypes are elicited in wild-type flies upon expression of RNAi constructs against CK2α/β, and that these defects are sensitive to *h* gene dosage. Together, these studies suggest that CK2 contributes to repression by Hairy.

Introduction:

Hairy is a basic helix-loop-helix (bHLH) repressor, which plays multiple functions during development (Ingham et al., 1985; Rushlow et al., 1989). During embryogenesis, its periodic expression drives segmentation via repression of genes such as *fushi tarazu* (Ish-Horowicz and Pinchin, 1987). Later, it regulates neurogenesis by repression of *achaete* or *atonal*, two bHLH proneural transcription activators (Brown et al., 1995; Ohsako et al., 1994; Van Doren et al., 1994). Hairy, the Enhancer of split and Deadpan bHLH proteins, along with their mammalian homologs, are referred to as the HES repressors. The precise patterning of gene expression, which is necessary for animal development, entails multiple mechanisms, and in this regard all HES proteins serve as transcriptional repressors (Fischer and Gessler, 2007). However, little information has been forthcoming on whether repression by Hairy is regulated by phosphorylation in vivo, and if so the identity of the protein kinase(s) that target it.

We describe studies that implicate protein kinase CK2 as a regulator of repression by Hairy during bristle patterning. CK2 is a highly conserved Ser/Thr protein kinase ubiquitous amongst eukaryotes (reviewed in Litchfield, 2003). Biochemical studies have shown that the catalytic CK2α subunit exhibits ~20% of the activity of the ($\alpha 2 \beta 2$) holoenzyme, while the regulatory CK2β subunit stimulates phosphorylation and modulates specificity (Bidwai et al., 1992a; Bidwai et al., 1993). CK2 modifies residues located within micro-acidic domains (Kuenzel et al., 1987) and targets proteins involved with transcription, cell-cycle progression and signal transduction (reviewed in Litchfield, 2003). Drosophila CK2 is structurally similar to its homologs in other organisms (Glover et al., 1983) also plays critical roles. Its activities are essential for embryogenesis (Jaffe et al., 1997), and for conserved and vital signaling pathways such as Notch (Karandikar et al., 2004; Trott et al., 2001). For example, CK2 phosphorylates E(spl)M5, M7, and M8 (Trott et al., 2001), the

downstream nuclear effectors of Notch signaling in neurogenesis. Consistent with multiple functions, null alleles of *CK2α* or *CK2β* elicit lethality (Jauch et al., 2002; Lin et al., 2002).

In this report, we characterize the biochemical and genetic interaction of CK2 and Hairy. Using in vitro phosphorylation and pull-down assays, we show that Hairy is a substrate and an interacting partner of the CK2 holoenzyme. Importantly, we show genetic interaction between mutants in *CK2* and *h*. Flies trans-heterozygous for alleles of *CK2α* and *h* display ectopic bristles (neural hyperplasia) and closely mimic the bristle phenotypes of homozygous mutations in *h*. Similar phenotypes are also elicited in otherwise wild type flies upon simultaneous knockdown of CK2α+β. Together, these studies identify CK2 as a new interacting partner of Hairy, and implicate a role for phosphorylation in the regulation of Hairy-mediated repression in vivo.

Results:

CK2 phosphorylates Hairy.

We first investigated the phosphorylation of Hairy using two purified isoforms of CK2. These are, the α2β2 holoenzyme from Drosophila embryos (CK2-HoloE), and monomeric CK2α (Fig. 1A). We find that GST-Hairy is robustly phosphorylated by CK2-HoloE, but weakly by CK2α (Fig. 1B). The weak phosphorylation of Hairy by CK2α does not reflect lack of activity, because this preparation efficiently phosphorylates the CK2-peptide (RRREEETEEE, Kuenzel et al., 1987), and is potently stimulated by the activator poly(DL)lysine (not shown). We tested for specificity and found that GST was not modified by CK2α or CK2-HoloE (Fig. 1B). The preferential phosphorylation of Hairy by CK2-HoloE, suggests that the regulatory CK2β subunit is important for this interaction.

To rule out the possibility that CK2-HoloE is contaminated with a kinase, other than CK2, and that this enzyme was responsible for phosphorylation of Hairy, we exploited the

observations that CK2 is inhibited by Heparin, and that ATP or GTP can serve as phosphoryl donors (Glover et al., 1983). We find that phosphorylation is strongly inhibited by 0.25 μg/ml of Heparin (Fig. 1C). Similarly, a 50% reduction in radiolabeling was observed with 50 μM cold-GTP (not shown), a concentration that correlates with the Km of CK2 (65 μM) for this nucleotide (Glover et al., 1983). Together, these results suggest that Hairy is a bona fide in vitro substrate of CK2.

Hairy directly interacts with CK2-HoloE.

We next assessed if Hairy forms a direct complex with CK2. Immobilized GST or GST-Hairy were used to pull-down CK2α or CK2-HoloE, and the bound and unbound fractions were analyzed by Western blots. As expected, neither CK2α nor CK2-HoloE interacted with free beads (Fig. 1D, lanes 1-4) or with GST-beads (Fig. 1D, lanes 5-8), consistent with our finding that neither isoform phosphorylates this affinity tag (see Fig. 1B). The faint bands marked with an asterisk in lanes 5 and 7 (see Fig. 1D) reflect weak crossreactivity of the CK2 antibody with GST, because their mobility is distinct from CK2β. Consistent with the phosphorylation analysis, CK2α weakly interacted with GST-Hairy beads (Fig. 1D, lanes 9 and 10). On the other hand, incubation of GST-Hairy beads with CK2-HoloE (Fig. 1D, lanes 11 and 12) resulted in significant amounts of CK2 in the pellet fraction, demonstrating that Hairy and CK2-HoloE form a direct complex. We estimate (by densitometry) that ≥60% of the available CK2-HoloE interacted with Hairy. Since CK2 was present at a low concentration (<5 μg/ml) and binding assays were conducted at physiological ionic strength (150 mM NaCl), complex formation appears to be efficient. These results show direct and preferential interaction of Hairy with CK2-HoloE. Consistent with a positive role for CK2β, we find that CK2-HoloE displays ~20-fold higher Vmax/Km for phosphorylation, as compared to CK2α (Fig. 1E).

CK2 genetically interacts with *hairy*.

We next tested for genetic interactions between *CK2* and *hairy* (*h*), as this would provide compelling in vivo evidence, and, perhaps, uncover the consequences of this phosphorylation. Aside from its roles in embryogenesis, Hairy regulates specification of the macrochaetes (MC's, the large bristles) and microchaetes (mc's, the small bristles), components of the fly peripheral nervous system (PNS). In this regard, the name-giving phenotype reflects the specification of excess MC's and mc's, as attenuated *h* activity elicits a greater density of these two bristle types (Rushlow et al., 1989). For these studies, we employed three alleles, i.e., h^1, h^2 and h^{41}. Of these, h^1 and h^2 are hypomorphic and, accordingly, ~70% of h^1 and h^2 animals are homozygous viable. In contrast, h^{41}, which terminates Hairy at Cys^{114}, is a strong loss of function and is homozygous lethal (Wainwright and Ish-Horowicz, 1992). As previously reported, h^1/h^1 or h^2/h^2 animals display ectopic MC's and mc's on the thorax, scutellum and the wing veins, phenotypes that are hallmarks of partial loss of *h* function (Fig. 2 and see below). We employed *Timekeeper* (*Tik*), a catalytically inactive allele of *CK2*α (Lin et al., 2002), which is homozygous lethal.

The Drosophila wing is characterized by six veins (L0-L6), and bristles at the anterior or posterior wing margins (AWM and PWM, Fig. 2B). In the wild type, bristles are devoid from the wing veins or the inter-vein region (Fig. 2B). In contrast, ≥99% of h^1/h^1 or h^2/h^2 flies display bristles along the L2 vein (Fig. 2C, D), to a lesser extent along L5, and interspersed in the inter-vein regions (not shown). These bristles are not seen in flies heterozygous for these alleles of *h* or *Tik* (Fig. 2E-H).

We conducted crosses between *Tik* and *h* alleles (Fig. 2A), and assessed for bristles on the wing veins. As Fig. 2 shows, bristles are manifest with high penetrance (85-89%) along the L2 vein in flies trans-heterozygous for any one of the three alleles of *h* in combination with *Tik* (Fig. 2I-K). In addition, these flies also displayed ectopic bristles (mc's) on the head and halteres (not shown). These phenotypes mimic those of h^1/h^1 or h^2/h^2 animals (Fig. 2C-D, and Rushlow et al., 1989). In all crosses, the percent viability of trans-

heterozygous progeny (h^1/*Tik*, h^2/*Tik*, or h^{41}/*Tik*) was similar to the controls, suggesting that halved dosage of CK2 is not rate limiting for embryonic functions of Hairy. No adult eye defects were observed, even though Hairy plays a role in eye development (Brown et al., 1995). The genetic interactions between *CK2*α and *h* suggest that modification of Hairy by CK2 is physiologically relevant for neurogenesis.

Compromising CK2 elicits MC defects that mimic partial *hairy* loss of function.

We next assessed whether compromising CK2 levels/activity in wild type flies will phenocopy the bristle defects observed in hypomorphic *h* backgrounds. We employed the Gal4-UAS system (Brand and Perrimon, 1993), to knockdown CK2, and assessed effects on the MC's using three UAS-constructs. Two are RNAi-constructs (*UAS-CK2*α*-RNAi* and *UAS-CK2*β*-RNAi*), while the third (*UAS-Tik*), encodes catalytically dead CK2α that behaves as a dominant-negative, i.e., a CK2-DN (Bose et al., 2006). Transgenes were expressed with the drivers, *hGal4* or h^{H10}*Gal4*. Importantly, the latter driver is homozygous lethal because the Gal4 insertion has rendered *h* non-functional (Huang and Fischer-Vize, 1996). Thus *hGal4* enabled us to assess the effects of compromised CK2 in a background wild type (+/+) for *h*, whereas h^{H10}*Gal4* assessed effects in a background with reduced (halved) *h* dosage.

The scutellum of wild type flies displays four MC's and the absence of mc's (Fig. 3A). In contrast, ~32% of h^1/h^1 animals display ectopic MC's and mc's on the scutellum (Fig. 3B). Similarly, expression of *CK2*α*-RNAi* or *CK2*β*-RNAi* by *hGal4* (Fig. 3C-D) elicited ectopic MC's in 31-42% of the flies. Expression of *UAS-Tik* by *hGal4* led to similar effects, but with lower penetrance (Fig. 3E). These phenotypes are sensitive to *h* dosage, because expression with h^{H10}*Gal4*, a background with reduced *h* activity (see above), elicited ectopic MC's with greater severity (42-62%, Fig. 2F-H). The bristle defects in flies heterozygous for *hGal4* or h^{H10}*Gal4* were ~5-9% (Fig. 3), indicating that these insertions do not elicit

significant levels of ectopic MC's. None of the UAS-insertions, by themselves, displayed ectopic MC's (Fig. 3), demonstrating that their expression (by *hGal4* or h^{H10}*Gal4*) was required for the MC phenotypes. The RNAi-constructs are target-specific; their effects are neutralized by co-expression of the corresponding CK2 subunits (Bose et al., 2006), and are mimicked by the CK2-DN construct (*UAS-Tik*) that is not expected to have non-target effects.

Knockdown of CK2α+β elicits ectopic mc's on the scutellum and wing veins akin to *h* mutants.

While RNAi against CK2α or CK2β elicited ectopic MC's, these flies did not exhibit ectopic mc's as in h^1/h^1 animals (compare Fig. 3B, F, G). We thus tested whether RNAi against CK2α+β would elicit ectopic mc's on the scutellum and the L2 vein, and mimic h^1/h^1 (Figs. 4A, 2C), but not h^2/h^2, $h^2/Tm3$ or $h^1/Tm3$ flies (Fig. 4B and inset). Similar to h^1/h^1 flies, RNAi against CK2α+β elicited ectopic mc's on the scutellum and wing veins (Fig. 4C-E). Moreover, these flies displayed mc's along the L2 and L5 veins, both also affected in h^{41}/Tik trans-heterozygous flies (Figs. 2K, 4F). Thus, knockdown of CK2α+β elicits ectopic mc's on the scutellum and along the L2 and L5 wing veins, phenotypes that closely mimic mutations in *h*. Together, these results provide strong evidence that attenuated levels of CK2 elicits phenotypes that mimic *h* loss of function in vivo.

Discussion:

The biochemical and genetic studies make a strong case that CK2 and Hairy interact directly, that the latter is a substrate for CK2 phosphorylation, and that reduction of CK2 elicits phenotypes akin to *h* mutants. Importantly, these MC/mc phenotypes are sensitive to *h* gene dosage. The correspondence of these defects to established phenotypes of *h* alleles strongly suggests that CK2 regulates repression by Hairy in vivo. This regulation involves

interaction of this repressor with both subunits of CK2. This is the first demonstration that repression by Hairy is regulated by CK2. While this interpretation is consistent with the biochemical and genetic studies, direct evidence for phosphorylation of Hairy in vivo is currently lacking. The restricted developmental context and the absence of suitable antibodies, preclude analysis of this modification in developing tissues, i.e., the wing imaginal disc, where specification of MC's and mc's occurs (see below).

Hairy contains CK2 recognition motifs.

A number of substrates of CK2 that have been identified demonstrate that the recognition consensus is S/T-x-x-D/E (Kuenzel and Krebs, 1985). Hairy contains two motifs (Fig. 4), both of which conserve an acidic residue at the critical n+3 position. It is noteworthy that both sites are virtually identical in the Hairy protein from virilis, a Drosophila species that diverged from melanogaster ~40-50 Myr ago (Beverly and Wilson, 1984). We, however, note that Site-1 appears sub-optimal for phosphorylation by CK2, given the presence of a Pro at the n+2 position. Nevertheless, phospho-amino acid analysis shows that Hairy is phosphorylated by CK2 only at Ser residues (data not shown).

Repression by Hairy.

As stated above, the roles played by Hairy in specification of the mc's are well known. During the pupal stage, Hairy functions as a pre-pattern factor to restrict expression of proneural transcription factors of the *Achaete-Scute Complex* (*ASC*), whose activities are necessary for specification of the mc's and MC's. This restriction is an important feature of neural patterning.

The onset of neurogenesis in Drosophila involves expression of the *ASC* activators, which confer neural potential upon groups of cells called the proneural clusters, PNC's (Calleja et al., 2002; Joshi et al., 2006; Modolell, 1997; Simpson et al., 1999). Later, a single cell from each PNC is selected as the sensory organ precursor (SOP), and undergoes asymmetric divisions to give rise to the cell types that characterize these sensory

organs (Jan and Jan, 1998). In this context, Hairy restricts *ASC* expression to precise regions of the wing disc, the primordial tissue for the adult notum and wings. Consequently, loss of Hairy leads to expanded expression of *ASC* and ectopic MC's/mc's. Our observations that hetero-allelic combinations of *CK2*α and *h*, or CK2α+β RNAi, mimic the bristle phenotypes of hypomorphic *h* alleles, provides strong evidence that repression by Hairy is sensitive to CK2 dosage.

While CK2 plays roles in the cell cycle (Hanna et al., 1995), it is unlikely that ectopic bristles would manifest in the event of cell cycle arrest. As stated above, bristle development requires three cell divisions of the SOP's. In this case, cell cycle arrest in the PNC's would elicit loss of the SOP's, and manifest as missing bristles. Thus the ectopic MC's/mc's cannot be reconciled with loss of cell viability. More likely, compromised CK2 leads to hypo-phosphorylation of Hairy and impaired restriction of *ASC* expression. This interpretation is consistent with the ectopic MC/mc phenotypes that we describe (Figs. 2, 3 and 4), and that increased dosage of *ASC* elicits excess PNC's, from which the SOP's and bristles arise.

Implications on repression by Hairy.

Our previous studies on the related bHLH repressor E(spl)M8 provide a potential answer. In the case of M8, phosphorylation by CK2 accentuates repression of the bHLH activator Atonal (Karandikar et al., 2004). We have previously proposed that phosphorylation stabilizes a conformation that is permissive for this antagonism. Although the three dimensional structures of E(spl) proteins remain unknown, the CK2-sites are in close proximity to the C-terminus, as is also the case with Hairy. The possibility thus arises that a conformational switch might exert a similar influence on Hairy-mediated repression. Our studies thus expand on the repertoire of binding partners of Hairy, and further implicate a role for CK2 in Hairy-mediated repression in vivo.

Materials and methods:

Purification of Drosophila CK2 and phosphorylation of Hairy:

Monomeric CK2α was purified as described (Bidwai et al., 1992a), and the CK2 holoenzyme (CK2-HoloE) was purified from embryos according to Glover et al (Glover et al., 1983). The V_{max} of CK2α is 0.4 μmol/min/mg and that of CK2-HoloE is 1.6 μmol/min/mg, values identical to those reported (Bidwai et al., 1993). GST-Hairy fusion protein was purified to homogeneity as described (Trott et al., 2001). Phosphorylation was carried out using 40 ng CK2α or CK2-HoloE and 2 μg of GST fusion protein in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 μM ATP, 5 μCi γ^{32}P-ATP in a volume of 40 μl, for 10 min at 25^{o}C, and terminated with 10 μl of 5x SDS-buffer (Bidwai et al., 1993). Proteins were separated by SDS-PAGE, stained with Coomassie, and the destained gels were exposed to Kodak XAR-5 film. Kinetic constants were determined as described (Bidwai et al., 1993).

GST pull-down assays:

Purified GST or GST-Hairy (2 μg) was bound to 25 μl of glutathione-Sepharose 4B beads overnight at 4^{o}C. The beads were washed with 1.5 ml Wash-buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 5% glycerol, 1 mM PMSF, and 0.1% Triton X-100) to remove unbound proteins. The beads, containing immobilized GST-fusions or beads alone as a mock control were incubated with 100 ng of purified CK2α or CK2-HoloE for 3 hrs at 4^{o}C. Unbound CK2 was recovered, and the beads were washed 3 times 5 min each with 0.5 ml. of Wash-buffer. Bead-bound and -unbound CK2 was analyzed by Western blots using anti Drosophila CK2 antibody (1:1000, Dahmus et al., 1984) and anti-rabbit IgG coupled to alkaline phosphatase (1:3000).

Phenotypic Analysis:

The Gal4 lines and *hairy* alleles were obtained from the Bloomington Stock Center. *UAS-CK2α-RNAi* and *UAS-Tik* flies have been previously described (Bose et al., 2006), while *UAS-CK2β-RNAi* flies were a gift of Rob Jackson (Tufts). Crosses were performed at 25^{o}C, and bristle images were acquired and collated in Adobe Illustrator.

Acknowledgement:

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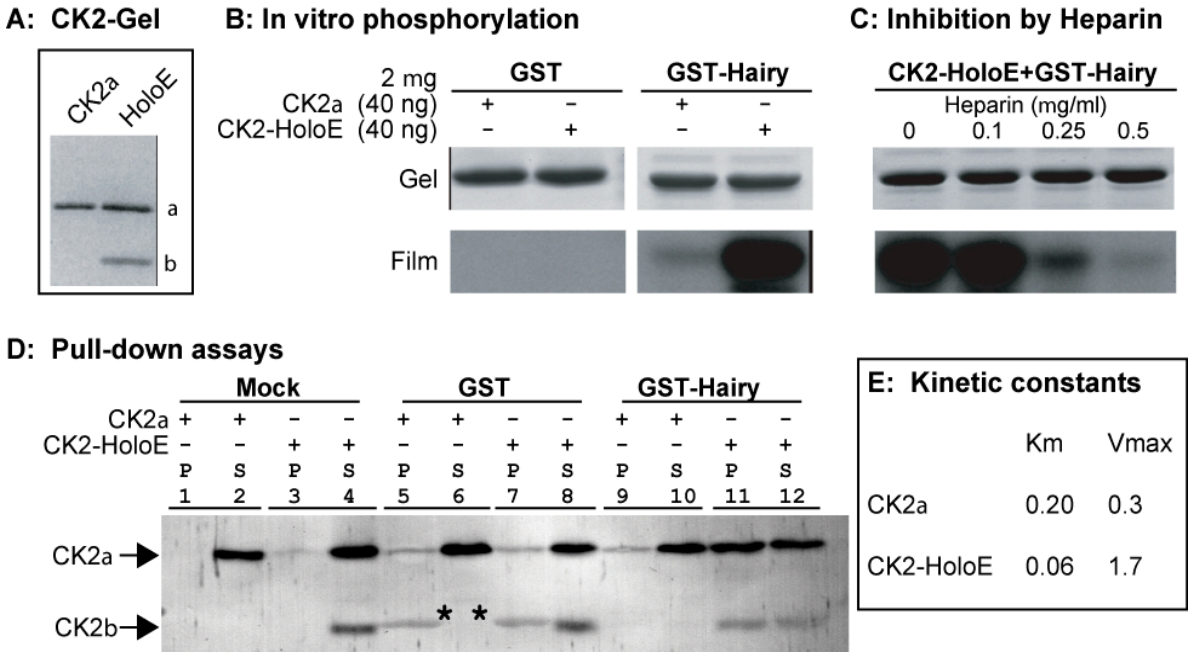

Fig. 1. CK2 interacts with and phosphorylates Hairy. (A) SDS-Page gel of purified CK2α and CK2-HoloE. (B) Phosphorylated proteins were separated (Gel) and autoradiographed (Film). (C) Hairy phosphorylation is inhibited by Heparin (D) CK2 pull-downs; lanes 1-4 (beads alone, mock), lanes 5-8 (GST-beads), and lanes 9-12 (GST-Hairy beads). CK2 in the bound (P, pellet) and the unbound (S, supernatant) fractions was assessed by Western blot. (E) Km and Vmax are reported as μM and nmoles/min/mg, respectively.

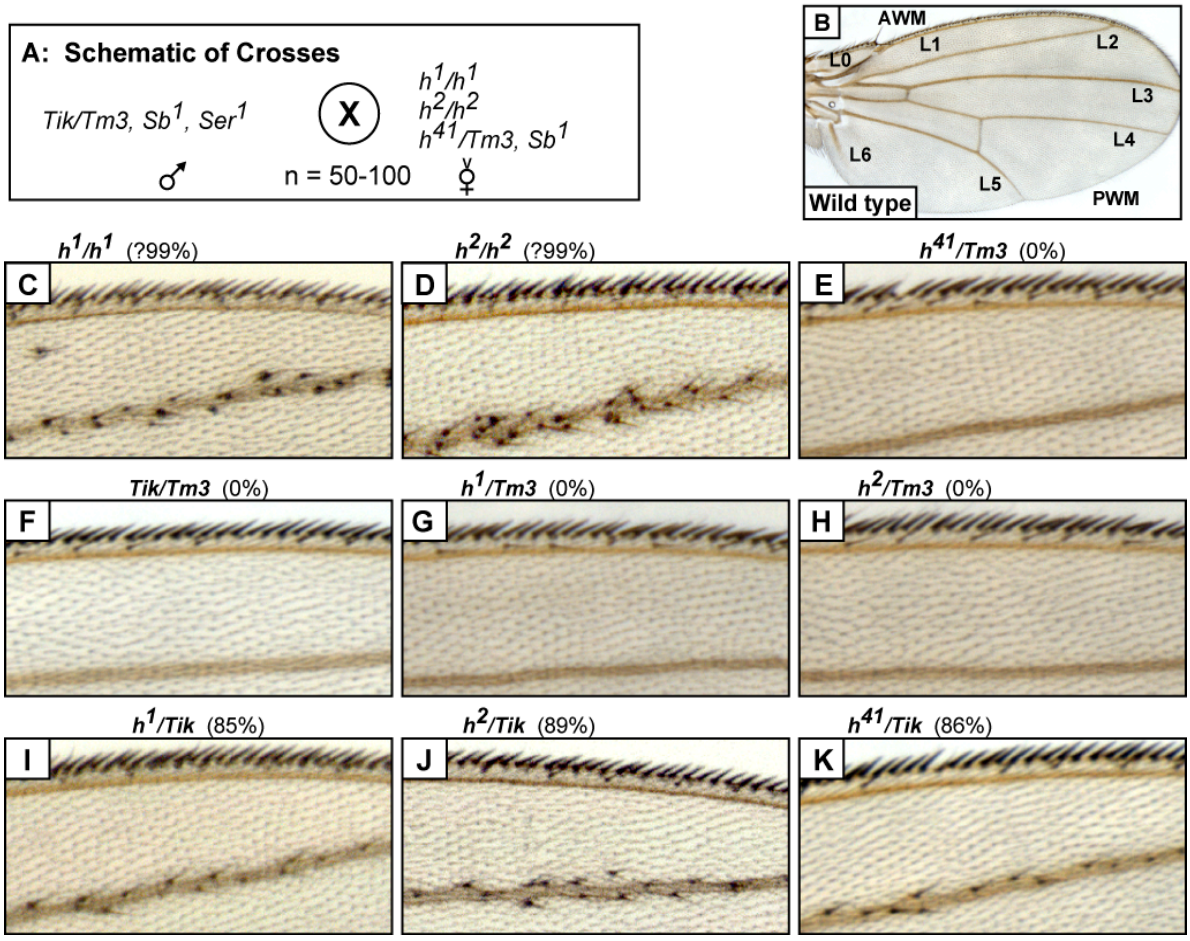

Fig. 2. CK2 genetically interacts with *h*. Alleles of *h* (h^1, h^2 and h^{41}) were tested for interactions with *Tik*, a CK2-DN allele. (A) Schematic of cross. Balanced stocks of *Tik/Tm3, Sb*^{1}*, Ser*^{1} were crossed to h^1/h^1, h^2/h^2 or *h*^{41}*/Tm3, Sb*^{1} flies. (B) Wild type wing showing the L0-L6 wing veins, anterior/posterior wing margin (A/PWM). (C-K) Wing L2 vein phenotypes. Numbers after genotypes indicate percent flies with L2-vein bristles.

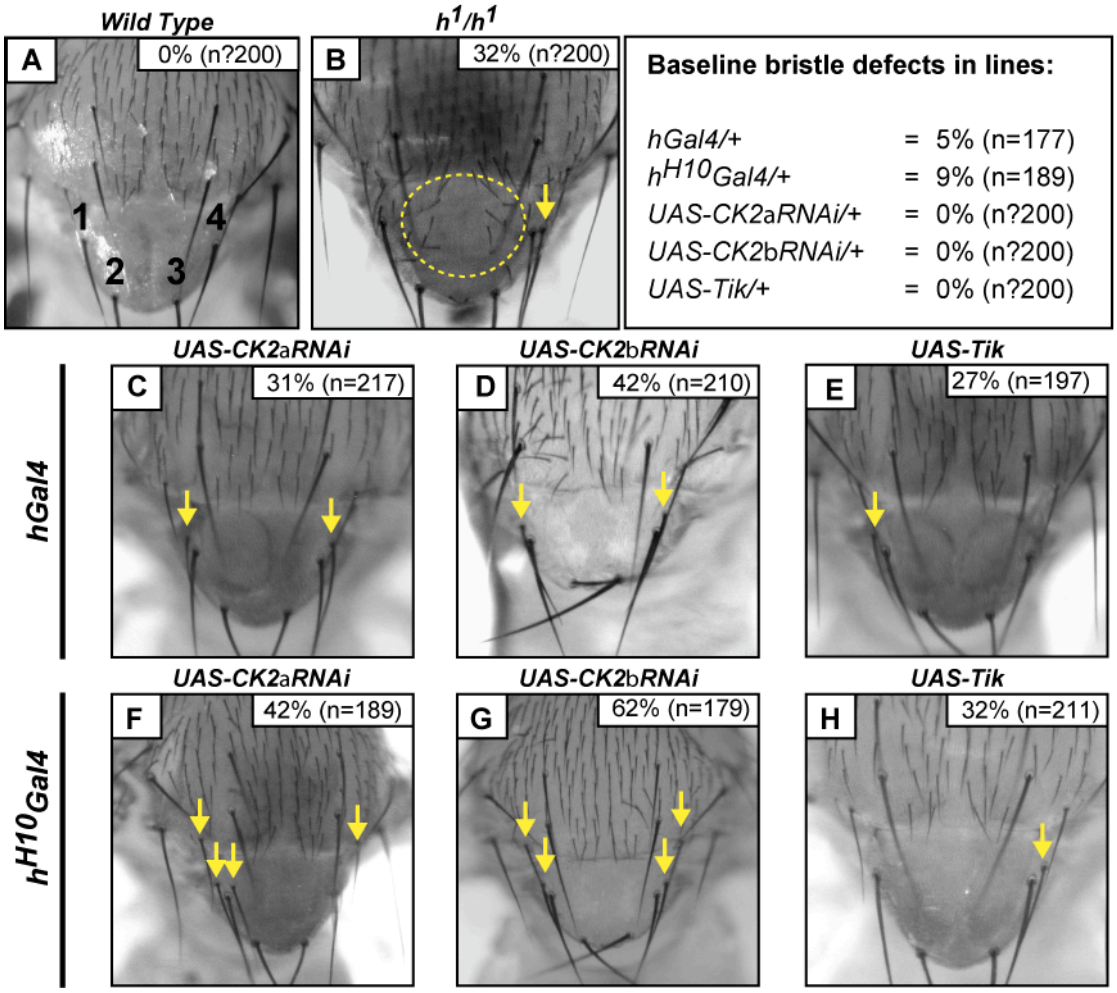

Fig. 3. Compromised CK2 elicits MC defects that are sensitive to *h* dosage. Scutellar MC and mc phenotypes of wild type (A) and h^1/h^1 flies (B). UAS-constructs were expressed with *hGal4* (C-E) or h^{H10}*Gal4* (F-H). Numbers denote percent flies with ectopic MC's (arrows). Ectopic mc's in h^1/h^1 flies are circled. Inset shows the bristle defects of relevant controls.

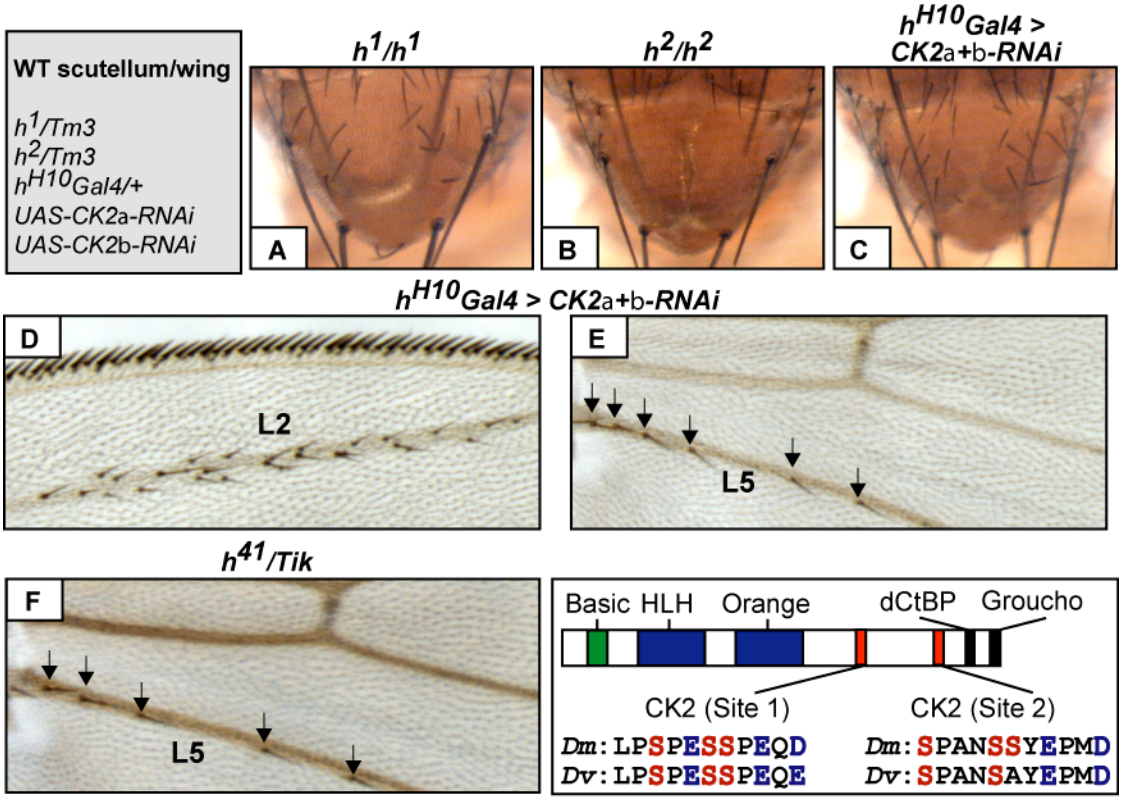

Fig. 4. Knockdown of CK2α+β mimics bristle defects of *h* mutants. RNAi against CK2α+β elicits mc's on the scutellum (C) akin to those in h^1/h^1 (A), but not in h^2/h^2 animals (B). Similarly, CK2α+β RNAi elicits ectopic bristles along the L2 and L5 veins (D-E) that mimic h^{41}/*Tik* flies (Fig. 2K and F). Inset shows genotypes that do not display ectopic mc's on the scutellum, or ectopic bristles along the L2 and L5 wing veins. (G) Schematic showing the CK2 sites in D. melanogaster (Dm) and D. virilis (Dv) Hairy.

Future perspectives:

The studies described in this dissertation indicate that multisite/hierarchical phosphorylation is required for E(spl)M8 activity. These modifications target a region of M8 that is conserved not only among different Drosophila species, but also in murine and human Hes6. In addition, the data indicates that multisite phosphorylation manifests during the process of lateral inhibition. This regulation appears to convert M8 from an autoinhibited condition to a state, which is competent for mediating repression. Given the conservation of the phosphorylation sites in mammalian Hes6, it is likely that a similar regulatory mechanism may well be applicable in case of mammals as well.

Future studies involving biochemical analysis will be required to further substantiate this multisite phosphorylation model. In addition, studies will be required to identify the MAPK involved in the regulation of M8. Along the same line, one important question that remains to be addressed is the regulatory mechanism(s) that control this phosphorylation cascade. Our data indicates that CK2 initiates this cascade. However, the mechanism(s) of how CK2 is regulated is unclear. CK2 is ubiquitously expressed, and does not appear to be regulated by second messengers. Given the fact that CK2 is involved in different temporal events, such as, circadian clock and cell cycle progression, suggests that the enzyme is tightly regulated. Based on this, it is reasonable to imply that CK2 is controlled by the availability of its substrates at a given time. However, our observation that co-expression of CK2 and M8 in the MF does not exhibit any loss of R8's, indicates otherwise.

Our observation that the reduced eye of M8S^{4}D, the CK2-mimetic variant, is sensitive to altered DER signaling raised the possibility that M8S^{4}D can provide a suitable background for 'screens' to identify novel factors involved in eye development. Genetic screens can be conducted to identify enhancers/suppressors of the reduced eye phenotype of flies expressing M8S^{4}D. Such modifier screens can be useful in identifying proteins that

mediate crosstalks. We have also provided direct *in vivo* evidence of the autoinhibition model. In this context, it will be important to identify the motif in M8 that mediates this interaction. The genetic interactions between *Tik*, the dominant negative *CK2*α allele, and alleles of *N, h* and *E(spl)* suggest that this allele provides a 'sensitized' background for screens to identify other retinal targets of this kinase.

It is anticipated that these studies will identify additional components, and provide better understanding of the mechanism(s) regulating repression by the E(spl)M8/Hes proteins. Given the evolutionary conservation of the phosphorylation sites, these studies may provide the basis for similar analysis with the related, but functionally distinct homologs, M5 and M7. Additionally, studies in Drosophila, a simpler model organism, may serve as the basis to understand the function/regulation of the mammalian Hes/Hey proteins, and provide new insights into the diseases associated with aberrant Notch signaling in humans.

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